Bottom-up and top-down herbivore regulation mediated by glucosinolates in *Brassica oleracea* var. *acephala*

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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

98 *vs.* low levels) (Gols et al. 2008; Harvey et al. 2011). It is known that cultivars of
99 *Brassica* species have low levels of glucosinolates in leaf tissue compared with wild
100 populations, which justifies the comparison between wild and cultivated species (Gols
101 and Harvey 2009). However, it is recognized that other plant traits, such as morphology,
102 phenology, primary chemistry and physiology, related to their different origins, life-
103 histories and genetic backgrounds, could also play a role in insect responses (Carmona
104 et al. 2011). At present, relatively few studies have used artificial selection to create
105 lines of brassicaceous plants with different glucosinolate profiles, and those that did
106 usually only had quantitative variation of sinigrin (Lankau 2007; Lankau and Strauss
107 2008; Lankau and Kliebenstein 2009). In the present study, we performed a divergent
108 selection from a local variety of kale, obtaining six plant genotypes which shared the
109 phenotype but which differed in glucosinolate patterns, having high or low content of
110 aliphatic (sinigrin and glucoiberin) and indole (glucobrassicin) glucosinolates. We
111 focused on insect responses to quantitative variation in glucosinolates (bottom-up
112 forces), by sampling a wide range of natural occurring specialist and generalist insect
113 herbivores. In particular, generalists were expected to be most sensitive to high sinigrin
114 concentration, as indicated in the literature (Ode 2006). On the other hand, the role of
115 plant chemistry in attracting natural enemies (top-down forces), such as parasitoids, was
116 assessed through the evaluation of parasitism rate. We also determined the extent to
117 which the cost of the synthesis of secondary defence metabolites is translated into
118 benefits (in terms of reduced herbivory), by assessing the variability of leaf damage
119 rates among plant genotypes. Finally, we investigated whether the differences between
120 plant groups in terms of herbivore abundance, the ratio between generalist and specialist
121 herbivores, and leaf damage rates could principally be explained by parasitism rate (top-
122 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 ºC for 40 days
163 and then transplanted into the field (Salcedo, NW Spain, 42º 24'N, 8º 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

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

On each sampling date, ten plants per row were thoroughly inspected in the search for the presence of insect herbivores on leaves. However, the eggs of *A. gamma*, *E. forficalis* and *P. xylostella* and the mining first instar larvae of *P. xylostella*, were not sampled due to their small size. Also, *M. brassicae* pupae were not sampled because they develop while buried in the soil. Plants inspected on one sampling date were always left untouched on the following date. All the lepidopteran species studied are, in Spain, bi- or multivoltine, and we therefore assumed that sampling would not significantly reduce their abundance in the plots. Field collected material (i.e. lepidopteran eggs, larvae and pupae, and *E. ornatum* eggs) was transported to the laboratory, identified, counted and placed in plastic 10cm-diameter-Petri-dishes, labelled with the collection date, host plant, block and insect identity. Larvae were reared individually in Petri-dishes and fed with fresh kale leaves. Lepidopteran immature stages were reared until adulthood or until the emergence of parasitoids. Eggs were maintained until larvae or parasitoids emerged. All rearing was carried out at room temperature (20 ± 2 ºC) and under a natural photoperiod. Because of the magnitude of the colonies of *B. brassicae* and *A. brassicae*, and of adults of *E. ornatum* and *P. cruciferae*, their abundance was estimated by using a subjective 0-4 rating scale (0 = absence; 1 = up to 5 individuals; 2 = up to 10; 3 = up to 50; 4 = more than 50). It was expressed as the average rating scale measured on 10 plants. The parasitism rate of *B. brassicae* and *A. brassicae* was not assessed because these hemipterans are more subject 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 ºC 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 ºC. 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 Phytoplan, Diehm & Neuberger GmbH, Heidelberg, Germany) and glucobrassicin (GBS, glucobrassicin potassium salt monohydrate, from Phytoplan, Diehm & Neuberger GmbH, Heidelberg, Germany) as an external standard and expressed in µmol 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= 263822 x (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

247 glucoiberin and high *vs.* low glucobrassicin) and general comparisons (i.e. between the
248 six genotypes), were also carried out. Insect count data were $\log_{10}(x+1)$ transformed
249 prior to the analyses, whereas the insect abundance estimated by rating scales and the
250 leaf damage level were arc-sin-square root transformed. Differences between means
251 were assessed by a LSD (Least Significant Difference) test. The larvae of *P. brassicae*,
252 *E. forficalis* and *A. gamma*, and the eggs of *P. rapae* were excluded from the statistical
253 analyses because they were only sporadically found.
254 Differences in the ratio of generalist (i.e. *M. brassicae*, *A. gamma*, *E. forficalis*)
255 to specialist (i.e. *P. xylostella*, *P. rapae*, *P. brassicae, E. ornatum*) herbivores among
256 plant genotypes and sampling dates (fixed factors) were assessed by using a Generalized
257 Linear Model with binomial proportion (logistic regression) and logit link function.
258 The influence of plant genotype, sampling date and the interactions between
259 them on the parasitism rate was assessed by means of Generalized Linear Model
260 (logistic regression) with binomial proportion and logit link function. The binomial
261 proportion (i.e. number of parasitized hosts/number of available hosts) was treated as
262 the response variable, whereas plant genotype and sampling date were the independent
263 variables. Pairwise and general comparisons among genotypes were assessed.
264 Parasitism rate was only assessed for immature stages of the lepidopteran species and *E.*
265 *ornatum* eggs.
266 In order to assess whether overall herbivore abundance, the ratio of generalist to
267 specialist herbivores and the leaf damage level were correlated with glucosinolate
268 concentrations or with the parasitism rate (fixed factors), a multiple linear regression
269 was adopted. Glucosinolate concentrations were expressed as the mean values for each
270 sample date (June and July in 2011, and July and October in 2012), with six replications
271 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

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

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

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

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

320 The size of the colonies of *B. brassicae* consistently increased in plants with low
321 glucobrassicin concentration and depended on the sampling date (pairwise comparisons)
322 (Table 2, Fig. 1-h). When considering general comparisons, only the sampling date and
323 the interaction between sampling date and plant genotype were found to be significant
324 for this species (Table 3).
325 The colonies of the whitefly *A. brassicae* (Fig. 1-i) and adults of the coleopteran
326 *P. cruciferae* (Fig. 1-l) varied their magnitude depending on the sampling date in both
327 pairwise (Table 3) and general comparisons (Table 3).
328 The ratio of generalist to specialist herbivores significantly differed among plant
329 genotypes (Wald test = 80, $P < 0.001$) and sampling dates (Wald test = 431.7 $P < 0.001$;
330 genotype × sampling date: Walt test = 313.54, $P < 0.001$). In particular, when both
331 years are considered together, plants selected for having high sinigrin and low
332 glucobrassicin content were seen to be significantly more visited by specialists than
333 generalists (online resource, Fig. S1-a, ESM). Considering the two years separately,
334 specialists dominated all plant groups in 2011, but preferred the genotype with high
335 sinigrin concentration (online resource, Fig. S1-b, ESM); in 2012, however, generalists
336 were more abundant, especially in plants with low sinigrin, and high or low glucoiberin
337 content (online resource, Fig. S1-c, ESM).
338 *Parasitoid responses to glucosinolate patterns*
339 Lepidopterans were parasitized by hymenopteran parasitoids belonging to the
340 Braconidae, Ichneumonidae, Encyrtidae, Pteromalidae, Eulophidae, Scelionidae and
341 Trichogrammatidae families, and by tachinid dipterans, whereas the eggs of the
342 hemipteran *E. ornatum* were attacked by *Trissolcus* sp. (Hymenoptera, Scelionidae) (see
343 Santolamazza-Carbone et al. 2013 for details of the parasitoid complex of *B. oleracea*
344 herbivores). In 2011, the parasitism rate experienced by the generalist herbivores was

345 lower (0.12 ± 0.01) than that experienced by the specialists (0.26 ± 0.03). A similar
346 trend was found in 2012 for generalists (0.15 ± 0.05) and specialists (0.41 ± 0.01).
347 The parasitism of *M. brassicae* larvae (Table 1), mainly exerted by the braconid
348 wasps *Microplitis mediator* L. and, occasionally, by *Cotesia rubecula* Marshall, was
349 significantly greater in those plants selected for high glucoiberin and high
350 glucobrassicin content (pairwise comparisons) (Table 4). When considering general
351 comparisons (Table 5), it was found that the parasitism rate was higher in genotypes
352 with high glucoiberin and high glucoibrassicin content (Table 1), and also affected by
353 sampling date, although the interaction was not significant
354 When performing pairwise comparisons, we found that plant genotypes did not
355 influence the parasitism rate of *M. brassicae* eggs, or of *P. rapae* and *P. xylostella*
356 larvae (Table 1 and 4). Similar results were obtained from general comparisons of plant
357 genotypes for the parasitism rate of *M. brassicae* eggs (Table 5). Sampling date did
358 significantly affect the parasitism rate of *P. rapae* larvae (general comparisons), but the
359 interaction between genotype and sampling date did not (Table 5). The parasitism rate
360 of *P. xylostella* larvae was not affected by the plant genotype, the sampling date or their
361 interactions when considering both pairwise (Table 4) and general comparisons (Table
362 5).
363 In 2011, 13.5% of *E. ornatum* eggs were parasitized, and parasitism rate was
364 highest in plants with low sinigrin and high glucobrassicin content (Table 1 and 4). In
365 2012 we did not detect any parasitism (Table 1). Further analysis (general comparisons)
366 confirmed the positive effect of high glucobrassicin plants on the egg parasitism rate of
367 this hemipteran, as well as the importance of the sampling date (Table 5).
368 *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.

394 This means that the role of specialist hemiptera on altering the net value of plant
395 defence traits could be crucial, as also reported by Lankau (2007). Furthermore, the
396 third trophic level represented by hymenopteran and dipteran parasitoids also performed
397 differently depending on the plant genotype, which is likely to be due to variation in
398 both the host density and the volatile blends emitted by the host plants (Gols and
399 Harvey 2009).
400 *Herbivore responses to glucosinolate patterns*
401 Host plants with high sinigrin and low glucobrassicin concentrations harboured a
402 significantly higher load of specialists. In particular, lepidopterans were outnumbered
403 by specialist hemipterans in 2011, especially in plant groups with high sinigrin content,
404 although this did not occur in 2012. There are a number of reasons that may explain the
405 wide distribution of the hemipterans across the plant genotypes: for example, insects
406 may build up high numbers on preferred host plants, eventually spilling over onto less
407 preferred ones (associational susceptibility) (White and Whitam 2000). Furthermore,
408 variation in the ratio between generalists and specialists also depended on the temporal
409 components of herbivory: generalist species tended to avoid plants previously
410 consumed by specialists, whereas specialists often colonized in large numbers plants
411 that were already being consumed by other phytofagous insects, irrespective of their
412 feeding mode (Poelman et al. 2010).
413 As expected, egg and larval abundances of *M. brassicae* were lower in plants
414 with high sinigrin concentration. Attraction of the *M. brassicae* female to patches of
415 plants may be mediated by visual and biochemical cues, while the decision of where to
416 oviposit within a plant population would depend on the specific plant chemistry. The
417 response of *M. brassicae* larvae to glucoiberin is less obvious. On the one hand, larvae
418 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

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

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

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

469 pattern of different *Brassica* crops did, however, highlight the importance of having a
470 high content of aliphatic compounds, such as progoitrin, epiprogoitrin, gluconapin and
471 glucoraphanin, and of the indole glucobrassicin in order to reduce the extent of damage,
472 although the effects also depended on plant age (Bohinc et al. 2013).
473 *Parasitoid responses to glucosinolate patterns*
474 Parasitization of insect hosts is the result of a complex process which involves the
475 attraction of parasitoids by semiochemical cues emitted by both the host (kairomones)
476 and the plant (synomones), parasitoid arrestment and host searching, host selection,
477 acceptance and oviposition (Godfray 1994). In the present study, the proportion of
478 herbivores that were parasitized was affected by differences in the herbivore-induced
479 volatile emissions of the different plant genotypes. Herbivore density was not
480 manipulated because we were also interested in measuring herbivore abundance on
481 different plant genotypes, and investigating its impact on the parasitism rate. However,
482 the host density-dependent effect was not seen to contribute to top-down control. *E.*
483 *ornatum* eggs, for example, were more abundant in plants with high sinigrin content,
484 but suffered from a higher parasitism rate in plants with low sinigrin content. *M.*
485 *brassicae* larvae were more abundant in plants with low glucoiberin content but not
486 more parasitized there. Similarly, *M. brassicae* larvae and *E. ornatum* eggs were more
487 parasitized in plants with high glucobrassicin content, even though their populations
488 were not especially high on this genotype. These findings agree with work carried out
489 on other *B. oleracea* varieties, which has shown volatile indole-derivates to be
490 important in the attraction of natural enemies of pentatomid bugs (Conti et al. 2008). In
491 fact, egg oviposition by herbivores could induce specific plant responses which are
492 relevant for host location by egg parasitoids (Fatouros et al. 2012).
493 *Top-down vs. bottom-up effects*

494 Whether or not plant resources, natural enemies, or both, determine the abundance of
495 insect herbivores in natural multi-trophic systems has long been a topic of debate
496 (Hunter and Price 1992; Halaj and Wise 2001) that is yet to reach a general consensus.
497 In comparison with natural habitats, managed crop systems have a relatively simple
498 food web structure, characterized by the presence of a homogeneous plant community,
499 for which the impact of bottom-up and top-down forces appears more predictable. The
500 higher propensity of crop habitats to experience strong trophic cascades was clearly
501 shown through meta-analysis by Halaj and Wise (2001). In this type of habitat the
502 reduction of predator abundance, more than plant chemical defences, generally
503 increased herbivory and reduced primary plant production (Halaj and Wise 2001). Our
504 results, however, indicate the opposite trend. In our study system, the impact of bottom-
505 up forces on insects agrees with the general view about the importance of plant
506 mediated forces in terrestrial ecosystems (Denno et al. 2002). Furthermore, previous
507 studies focused on brassicaceous plants (Newton et al. 2009b; Kos et al. 2011a) have
508 also highlighted that plant chemistry and morphology have more impact than natural
509 enemy activity in shaping herbivore abundance.
510 The statistical analyses showed that glucosinolate concentrations have a
511 significant effect on the overall herbivore abundance, on the relative abundance of
512 generalist and specialist herbivores, on the parasitism rate and on the leaf damage rate.
513 On the other hand, parasitism rate also contributed in shaping the leaf damage rate in
514 2011, and the ratio between generalist and specialist insects in 2012, which suggests
515 that in reality many ecological forces combine to determine the patterns observed in the
516 field. Interestingly, in 2011 the increase in sinigrin content was positively correlated
517 with herbivore abundance, whereas an opposite trend was found for leaf damage rate.
518 The massive presence of the hemipteran *E. ornatum*, and in particular the preference

519 manifested by ovipositing females for plants with high sinigrin content, explained the
520 positive relationship between sinigrin concentration and herbivore abundance. The
521 reduction in leaf damage, on the other hand, can be explained by the fact that although
522 overall herbivore numbers increased with high sinigrin content, the numbers of
523 generalist lepidopteran herbivores decreased. This prompted a net decline in overall leaf
524 damage because the damage provoked by chewing insects (including Lepidoptera) was
525 disproportionately high and more widely detected than damage from hemipteran sap-
526 sucking species. In 2012, the leaf damage rate in plants with high sinigrin content was
527 also reduced as a result of the increase in parasitism rate among the lepidoptera (the
528 same pattern was not seen in hemiptera: *E. ornatum* eggs, for example, were parasitized
529 in 2011 but not in 2012). These findings highlighted the role of sinigrin in plant
530 protection. However, an ecological cost of plant defence through sinigrin synthesis
531 does exist, because specialists were more attracted to a high concentration of this
532 secondary metabolite. Previous field studies on the role of *B. oleracea* chemicals on
533 insect herbivore biodiversity showed the impact of glucoiberin in shaping insect
534 communities (Poelman et al. 2009; Kos et al. 2011a). Data supporting the idea that
535 sinigrin influences herbivore choices comes from laboratory trials (Shields and Mitchell
536 1995; Gols et al. 2008) and from field tests performed in controlled environments (by
537 manipulating insect presence and abundance) (Lankau 2007; Lankau and Kliebenstein
538 2008; Lankau and Strauss 2009; Kos et al. 2011a). This is the first time that an
539 investigation under natural conditions reported significant responses to sinigrin,
540 glucoiberin and glucobrassicin expressed by herbivores with different feeding modes
541 and behavioural ecologies, and by their parasitoids.

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

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

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