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Variation and fitness costs for tolerance to different types of herbivore damage in *Boechera stricta* genotypes with contrasting glucosinolate structures

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Summary

• Analyses of plant tolerance in response to different modes of herbivory are essential to an understanding of plant defense evolution, yet are still scarce. Allocation costs and trade-offs between tolerance and plant chemical defenses may influence genetic variation for tolerance. However, variation in defenses also occurs for the presence or absence of discrete chemical structures; yet, the effects of intraspecific polymorphisms on tolerance to multiple herbivores have not been evaluated.

• Here, in a glasshouse experiment, we investigated the variation for tolerance to different types of herbivore damage, and direct allocation costs, in 10 genotypes of *Boechera stricta* (Brassicaceae), a wild relative of *Arabidopsis*, with contrasting foliar glucosinolate chemical structures (methionine-derived glucosinolates vs glucosinolates derived from branched-chain amino acids).

• We found significant genetic variation for tolerance to different types of herbivore. Structural variations in the glucosinolate profile did not influence tolerance to damage, but predicted plant fitness. Levels of constitutive and induced glucosinolates varied between genotypes with different structural profiles, but we did not detect any cost of tolerance explaining the genetic variation in tolerance among genotypes.

• Trade-offs between plant tolerance to multiple herbivores may not explain the existence of intermediate levels of tolerance to damage in plants with contrasting chemical defensive profiles.

Introduction

Plants possess a rich diversity of defensive adaptations against herbivores, which may enable resistance to herbivore attack or tolerance to damage. In the first case, plants rely on traits that reduce herbivore damage on plant tissues, such as trichomes, spines or toxic secondary compounds (reviewed in Strauss & Zangerl, 2002). Alternatively, some plant genotypes may be less susceptible to negative impacts when herbivore damage occurs (Strauss & Agrawal, 1999). In both cases, plant defensive traits are typically genetically complex quantitative traits (Rosenthal & Kotanen, 1994; Weinig *et al.*, 2003; Agrawal & Fishbein, 2006; Schranz *et al.*, 2009), and often show heritable variation within and among populations (e.g. Fornoni & Nuñez-Farfán, 2000; Kliebenstein *et al.*, 2001; Windsor *et al.*, 2005; Løe *et al.*, 2007). In particular, plant tolerance to damage (the ability to regrow and reproduce after herbivory; Strauss & Agrawal, 1999) is genetically variable in many species (reviewed by Strauss & Agrawal, 1999; Fornoni *et al.*, 2003; Nuñez-Farfán *et al.*, 2007; but see Ivey *et al.*, 2009), and therefore may evolve in response to natural selection. However, the ecological and genetic basis of tolerance variation is still not well understood, especially among populations (Fornoni *et al.*, 2003).

The expression of plant tolerance to damage varies among environments (Wise & Abrahamson, 2007). Resource availability, the timing and magnitude of damage, and the type of herbivore damage are important ecological factors influencing the evolution of tolerance (e.g. Maschinski &

Whitham, 1989; Strauss & Agrawal, 1999; Tiffin, 2002; Steven *et al.*, 2007; Suwa & Maherali, 2008). In particular, tolerance to damage is often dependent on the type of tissue damaged – that is, apical vs foliar damage, damage on young leaves vs mature ones, or damage to roots vs leaves (e.g. Houle & Simard, 1996; Stinchcombe, 2002; Boalt & Lehtilä, 2007; but see Tiffin & Rausher, 1999). However, these studies simulate damage mainly through manual clipping, which may be a poor surrogate for genuine herbivory (Strauss & Agrawal, 1999). In addition, little is known about how different kinds of herbivore damage influence tolerance to herbivory across naturally varying genotypes (but see Agrawal *et al.*, 1999; Tiffin & Rausher, 1999; Pilson, 2000). However, to understand the evolution of tolerance, it is essential to determine whether conspecific genotypes show differential tolerance to herbivore damage. Furthermore, plants are often attacked by multiple herbivore species, and so we must examine diverse generalist and specialist herbivores feeding on different parts of the plant. In addition, plants face different types of herbivore across their ranges, which may result in different ecological and evolutionary outcomes (Thompson, 1988; Stinchcombe & Rausher, 2002; Strauss & Irwin, 2004).

The influence of quantitative intraspecific variation in plant chemical defenses on tolerance to damage is frequently studied in the context of ecological trade-offs and direct costs of tolerance (Strauss *et al.*, 2002; Leimu & Koricheva, 2006). Allocation costs occur when there are significant negative genetic correlations between tolerance and resistance, or between tolerance and fitness in the absence of herbivores. Such costs are thought to maintain the existing levels of genetic variation in tolerance within species (Strauss & Agrawal, 1999; Strauss *et al.*, 2002), but they are not always detected (Leimu & Koricheva, 2006). Both tolerance to damage and the magnitude and significance of tolerance costs may depend on phenotypic plasticity, such as differences in trait expression through ontogeny (Boege *et al.*, 2007; Barton, 2008) or in levels of defense induction (Agrawal, 1998, 1999). However, genetic variation in chemical defenses occurs not merely at the quantitative level, but also with regard to the presence or absence of discrete chemical structures (e.g. Schranz *et al.*, 2009), and differential allocation costs could arise among genotypes with different chemical compositions, especially if geographical structure underlies such variation. Recent studies have shown that constitutive structural polymorphism in chemical plant defenses affects plant resistance to herbivores and influences herbivore communities (Newton *et al.*, 2009; Schranz *et al.*, 2009); yet, to our knowledge, the effects of intraspecific chemical polymorphism on plant fitness have not been evaluated in the context of herbivore tolerance. However, if distinct defensive compounds have different biosynthetic costs, such structural polymorphisms in plant chemical defenses may also influence tolerance or its costs (Koricheva, 2002 and references therein).

Here, we investigate genetic variation in tolerance to different types of damage and the allocation costs of tolerance to damage in genotypes of *Boechera stricta* (Brassicaceae) with contrasting foliar glucosinolate chemical structures. Tolerance is defined here as the difference in fitness between damaged and undamaged plants (Strauss & Agrawal, 1999). In *B. stricta*, heritable natural polymorphism exists in aliphatic glucosinolates within and among populations (Schranz *et al.*, 2009). Although *Arabidopsis*, *Brassica* and most other crucifers produce leaf glucosinolates largely derived from the amino acids methionine or tryptophan, some genotypes of *B. stricta* synthesize glucosinolates from the branched-chain amino acids (BCAA) valine, leucine or isoleucine (Windsor *et al.*, 2005; Schranz *et al.*, 2009). Although there is currently little information about the ecological role of BCAA-derived aliphatic glucosinolates, recent quantitative trait locus (QTL) mapping has shown that heritable resistance to the larvae of the generalist lepidopteran *Trichoplusia ni* (Noctuidae) varies significantly between genotypes with contrasting glucosinolate profiles (Schranz *et al.*, 2009). In particular, lines producing methionine-derived glucosinolates were significantly more resistant and suffered less leaf herbivory than lines producing predominantly BCAA-derived glucosinolates (Schranz *et al.*, 2009). In addition, biosynthesis of BCAA and methionine-derived glucosinolates is controlled by different genes using different metabolic pathways (Mikkelsen & Halkier, 2003), which may affect tolerance if differences in the biosynthetic costs of such compounds vary.

To our knowledge, ours is the first investigation of the effects of natural structural polymorphism in plant chemical defenses on tolerance to different herbivores. Specifically, we address the following questions. Is there genetic variation in *B. stricta* for tolerance to herbivory? If so, does this variation depend on the type of herbivore damage or the glucosinolate structural profile (glucosinolates derived from methionine vs BCAA). Do allocation costs explain the observed genetic variation in tolerance to damage in *B. stricta*?, Is the magnitude and significance of tolerance costs determined by the type of herbivore damage, the structural glucosinolate profile or glucosinolate induction? We seek to determine the ecological and evolutionary significance of nonmethionine-derived aliphatic glucosinolates in plant defense to herbivory.

Materials and Methods

Study system

Boechera stricta (Graham) (previously *Arabis drummondii*) is a morphologically and genetically well-defined, monophyletic, short-lived perennial herb distributed across diverse

habitats in western North America (Mitchell-Olds, 2001; Song *et al.*, 2006). *Boechera stricta* is a predominantly self-fertilizing, sexual diploid, and is attacked by a wide array of specialist (e.g. the pierid *Pontia* spp.) and generalist (e.g. noctuids, grasshoppers, flea beetles and weevils) insect herbivores. In the field, levels of individual plant damage range between 0% and 100% (average damage per leaf, 8.8%; average proportion of leaves damaged, 13.1%), and there is substantial variation in the average herbivore damage among populations and years (T. Mitchell-Olds, unpublished). Plants produce between one and five inflorescences in late spring, and both fruit maturation and seed set take place in June–July.

Like other members of the Brassicaceae, *B. stricta* produces glucosinolates, which constitute a primary chemical defense against herbivores (Hopkins *et al.*, 2009). There is extensive natural genetic variation for type and quantity of glucosinolates within and among natural populations (Windsor *et al.*, 2005; Schranz *et al.*, 2009). The glucosinolate polymorphism controls allocation to BCAA- vs methionine-derived glucosinolates and predicts levels of herbivory (Schranz *et al.*, 2009).

Because a large portion of genetic polymorphism in *B. stricta* is distributed among populations (Song *et al.*, 2006), we examined one genotype from each population, in order to maximize genetic variation for a given sample size. We considered nine genotypes from our study areas in the Northern Rocky Mountains (see later and Supporting Information Fig. S1). One of these genotypes (Lost Trail, Montana) has been used as a parent for QTL mapping of insect resistance (Schranz *et al.*, 2009); hence, the other parent (Taylor River, Colorado) was also included for comparison.

Experimental procedure

Mature seeds of 10 genotypes were collected from 10 different populations located in the Rocky Mountains in the western USA (in Montana, Idaho and Colorado, see Fig. S1 and Table S1 for details). These populations are diverse in terms of ecological conditions and also differ in the levels of damage received by the plants. Genotypes included in the experiment were selected on the basis of their chemical background: four genotypes produce mainly BCAA-derived glucosinolates, and six genotypes produce methionine-derived glucosinolates (Table S1). We minimized potential maternal effects by using seeds from a second generation of self-fertilized, glasshouse-grown plants.

In November 2007, we placed 12 self-sib seeds/genotype into Petri dishes at 4°C for 6 wk of cold stratification. Once germinated, 11 seeds per genotype were individually planted and grown on standard soil (Fafard 4p mix; Fafard Inc., Agawam, MA, USA) in a randomized complete block design, with the blocks consisting of one tray of 40 ($5 \times 5 \times 6$ cm^3) pots, distributed randomly within the tray. The trays were placed in the Duke University glasshouse under controlled growth conditions. After *c.* 6 wk, plants were moved to a growth chamber (22°C, 16 h light and 8 h dark) and randomly assigned to the following four treatments: (1) undamaged control; (2) specialist herbivore treatment, with 33% of plant leaf area damaged by a caged second-instar *Pieris rapae* larva (Lepidoptera: Pieridae); (3) generalist herbivore treatment, with 33% of plant leaf area damaged by a caged second-instar *Trichoplusia ni* larva (Lepidoptera: Noctuidae); (4) manual clipping treatment, with 33% of each leaf clipped and removed using scissors. Therefore, each of the 10 genotypes had 11 individuals (replicated in blocks) in each of the four treatments, for a total of 440 plants.

The insect species used here are not native enemies of *B. stricta*, but have been used extensively to investigate plant functional responses to herbivory by specialist and generalist insects (e.g. Agrawal, 1999, 2000a; Jones *et al.*, 2006; Schranz *et al.*, 2009). *Pieris rapae* is able to detoxify glucosinolates but *Trichoplusia ni* is not, which affects the feeding behavior of the herbivores and the pattern of plant damage caused by each type of herbivore (see Notes S1 and Fig. S2). Second-instar *T. ni* larvae were ordered from Benzon Research Inc. (Carlisle, PA, USA) and fed on an artificial diet. Second-instar *P. rapae* larvae were obtained from a colony maintained in the laboratory on fresh *Raphanus sativa* leaves, originating from eggs provided by Carolina Biological Supply (Burlington, NC, USA). In both of the insect treatments, a single larva without any previous starvation period was placed on top of each plant. To guarantee a single insect on each plant, each plant–larva pair was enclosed using a cylindrical tube (diameter, 5 cm; height, 14 cm) made of acetate (3M®) with both ends open. One end was inserted into the soil and the other was covered by loose-weave fabric. Larvae were removed from the plants once 33% of the leaf area had been consumed (after 24–56 h and 24–88 h, for specialist and generalist insects, respectively). The size of the treated plants (estimated from the plant basal diameter) ranged between 3.8 and 11.4 cm. Plants were checked for damage 24 h after the infestation, and then every 8 h. In every census, we recorded both the proportion of leaves with herbivore damage and estimated the percentage of tissue removed per leaf (taken by the same person and ranging from 1% to 100%; see Schranz *et al.*, 2009 for a similar procedure) to calculate the percentage of plant damage. After 72 h, the majority of the plants (368 plants, 87.7%) in both insect treatments had the target level of damage, whereas 72 plants (12.3%) did not reach the level of plant damage desired or had excess damage. These plants were not included in the statistical analysis. More details on the feeding behavior of each insect species and the way that insects damaged the plants are given in Notes S1.

Like most perennial species, *Boechera* requires a cold vernalization period to induce flowering and seed production. For this reason, 1 wk after the herbivore treatments we moved the plants to a cold room (4°C, 16 h light and 8 h dark) for 6 wk, in order to initiate flowering and reproduction. This laboratory treatment provides an effective simulation of 'winter' vernalization because plants perceive vernalization cues only when temperatures are above freezing, rather than the long periods of below-freezing temperatures that occur in the wild. Subsequently, plants were moved back to the glasshouse (15.6–21°C, 16 h light and 8 h dark) until the end of the experiment (in May 2008).

Reproductive measurements

For each plant, we recorded both the flowering time (the number of days from germination until the opening of the first flower) and several correlates of maternal fitness: total number of flowers, seed set (total number of seeds/total number of flowers) and reproductive biomass [the weight of one individual seed randomly chosen (fresh weight using a Mettler Toledo® xs105 precision scale, Columbus, OH, USA) multiplied by the total number of seeds]. *Boechera stricta* is self-compatible and highly inbred (Song *et al.*, 2006); thus, differences in fitness are unlikely to reflect inbreeding depression.

Analysis of constitutive and induced glucosinolates

Concurrent with the tolerance experiment, we grew 440 additional plants from each of the same 10 genotypes under the same conditions for the analysis of constitutive and induced glucosinolates. The experimental procedure and methods for glucosinolate extraction, isolation, purification and quantification follow our previous methods (Schranz *et al.*, 2009) and are given in Notes S2.

Statistical analyses

To analyze the effect of herbivory on multiple fitness components, we conducted both multivariate analyses and general linear mixed models with maximum likelihood estimates, using JMP 7.0.1 (SAS Institute Inc., Cary, NC, USA). As fitness components were correlated (Table S2), we first performed a MANOVA to test the effects of treatment, genotype and their interaction on overall fitness. A significant interaction between genotype and treatment shows the existence of genetic variation in tolerance (i.e. difference in fitness between the damage treatments and the undamaged control, see the next paragraph) for overall plant fitness. Second, we conducted a principal component analysis (PCA) to obtain independent factors (after varimax normalized rotation) accounting for plant fitness traits. Factor scores correlated significantly with fitness were included as dependent variables in separate mixed models fitted to test the fixed effects of treatment, genotype and their interaction. We included plant size as a covariate and block as a random effect in these models. The interaction between plant size and genotype was nonsignificant (not shown) and was removed from the models. Reproductive values were log-transformed to improve normality and homoscedasticity.

Tolerance was estimated for each genotype and fitness component (i.e. principal component) as the difference in fitness between the damage treatments (either specialist herbivore, generalist herbivore or clipping) and the undamaged control (Strauss & Agrawal, 1999). Higher and positive values depict greater tolerance to damage than smaller or negative values. Damage levels and fitness components were on the same multiplicative scale (Wise & Carr, 2008). For each of the fitness components, genetic variation in tolerance was inferred from the significance of genotype by treatment interaction term in the linear mixed models described above. When a significant interaction between genotype and herbivore treatment was detected, we carried out tests of simple main effects using the SLICE option in JMP, which allows the effects of a given factor to be explored at each level of the other factors (Schabenberger *et al.*, 2000). In the context of this study, this test allowed us to determine, for each genotype, what type of herbivore damage had a significant effect on fitness components.

To analyze whether tolerance to damage is affected by the type of glucosinolate profile, we grouped the 10 genotypes into two categories: methionine-derived glucosinolates or BCAA-derived glucosinolates (see Table S1). Differences in tolerance means between these two groups were estimated using a nonparametric Kruskall–Wallis test with genotype as the unit of replication. In addition, to test the effect of the chemical background of the genotypes and herbivory on plant fecundity, for each fitness component, we fitted a general linear mixed model including treatment, the glucosinolate group (BCAA-derived vs methionine-derived) and their interaction as fixed factors, and block as a random factor. As plants with a high proportion of BCAA-derived glucosinolates were significantly larger (basal radius mean ± 1SE: BCAA-derived, 73.75 ± 1.98 mm; methionine-derived, 70.77 ± 1.89 mm; ANOVA: $F_{1,406} = 2.45$, $P = 0.015$), plant size was included as a covariate.

When significant genetic variation was found, we estimated the allocation costs of tolerance to damage. We analyzed the genetic correlation among genotype fitness means (in all cases, means are model-adjusted LS-MEANS) for damaged and undamaged plants using correlation analyses in JMP. A significant negative correlation between the fitness of damaged and undamaged plants indicates a cost of tolerance (Strauss & Agrawal, 1999). In addition, we analyzed the genetic correlation among genotype mean tolerance in each of the herbivore damage treatments (i.e.

tolerance to generalist vs tolerance to specialist damage; tolerance to generalist vs tolerance to clipping damage; and tolerance to specialist vs tolerance to clipping damage). Similarly, a significant negative correlation between tolerance to different types of damage would indicate a cost of tolerance.

Because all damaged plants had equal percentage of leaf area removed (see the section entitled 'Experimental Procedure'), we could not infer directly heritable variation for resistance among our plant genotypes. However, given the defensive role played by glucosinolates (at least against generalist herbivores, e.g. Hopkins *et al.*, 2009), for each type of damage, we also analyzed tolerance–chemical defense trade-offs by regressing the genotype tolerance means obtained for each type of damage and the genotype total glucosinolate concentration means at both the constitutive and induced level. A significant negative genetic relation between tolerance and the concentration of defensive metabolite indicates the presence of an allocation trade-off between tolerance and resistance (Leimu & Koricheva, 2006). Because the total concentration of constitutive and induced glucosinolates was affected significantly by the chemical background of the genotypes (see the Results section), we explored the covariance between tolerance and resistance through both groups. Because our data did not satisfy the assumptions of ANOVA (e.g. small sample size, non-normal errors and presence of outliers), we used a robust analysis of covariance based on M estimation (Chen, 2002). Robust ANCOVAs were then performed using the procedure ROBUSTREG in SAS 9.2 (SAS Institute Inc., Cary, NC, USA). In these analyses, genotypic mean tolerance was always the dependent variable and the glucosinolate group was the grouping factor. We included as covariates the mean genotype total glucosinolate concentration in the undamaged treatment (constitutive resistance) or the difference in the mean total glucosinolate concentration after 24 h of herbivory between damaged and undamaged plants (induced resistance). The statistical significance of ANCOVAs came from R_n^2 — robust Wald's linear test (Chen, 2002).

Results

Effects of herbivore treatments on overall plant fitness

A MANOVA conducted on all fitness components did not show a significant main effect of herbivore treatment (Table 1). However, there were significant effects of genotype on overall fitness, and the genotype by herbivore interaction (Table 1), which suggests that the magnitude and/or sign of the herbivore treatment on fitness depended on genotype. Analyses conducted separately on each of the fitness components (see the 'Results' section) revealed that herbivory affected the early fitness components, although its effect was dependent on genotype. Block and plant size also influenced plant fitness (Table 1).

Table 1 MANOVA to test the effects of genotype, herbivore treatment and their interaction on four fitness components of *Boechera stricta*

Source	Wilks' λ	df	*F*	*P*
Genotype	0.387	36, 1227	35.29	< **0.0001**
Herbivory	0.945	12, 865	1.54	0.1050
Genotype × herbivory	0.654	108, 1300	1.36	**0.0100**
Plant size	–	4, 327	8.22	< **0.0001**
Block	0.51	40, 1242	6.13	< **0.0001**

Significant values ($P < 0.05$) are in bold.

Fitness components and genetic variation in tolerance

Taken together, three independent factors accounted for 98.6% of the variation in reproductive traits in our PCA (Table S3). The first factor (PC1) depicts late plant fecundity, because it is related to seed set and reproductive biomass (Table S3). The second factor (PC2) indicates early fecundity, and is closely related to the numbers of flowers (Table S3), providing the opportunity for reproductive fitness via male function. Both factors are related to variation in fecundity, where higher and positive values indicate higher levels of seed and flower production. The third factor (PC3) depicts flowering time variation (Table S3). In this case, negative and smaller values denote early flowering times, and positive and higher values depict late flowering times.

Herbivory, as a main effect, did not influence the PC3 fitness component related to flowering time PC3 (Table 2). However, there was a significant effect of genotype, and the genotype by herbivore treatment interaction, on the PC3 fitness component (Table 2). This result suggests that the consequences of different types of herbivory on the PC3 fitness component are genotype dependent, and that there is significant genetic variation in tolerance when fitness components correlated with flowering time are considered (Fig. 1). Tests of main effects showed that six of 10 genotypes were not affected by herbivore treatment (Table 3). However, ID70 and MT49 genotypes flowered more rapidly in response to insect damage, whereas MT55 and SAD12 genotypes flowered more slowly in response to damage (Fig. 1).

Early fitness component PC2 differed significantly among genotypes and showed only marginal effects of herbivore treatment; however, the genotype by herbivore treatment interaction was significant (Table 2). This indicates that the fitness consequences of herbivore treatments depend on genotype, and there is significant genetic variation in tolerance for early fitness components (Fig. 2). Tests of main effects showed that most of the genotypes (seven of

Table 2 Summary results of the general linear mixed model testing the effects of herbivore treatment, genotype and their interaction on three principal components summarizing fitness-related variation in *Boechera stricta*

	PC1			PC2			PC3		
Fixed effects	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Genotype	9, 333	11.24	< **0.0001**	9, 332	14.52	< **0.0001**	9, 330	171.97	< **0.0001**
Herbivory	3, 331	2.38	0.069	3, 331	2.36	0.071	3, 330	0.81	0.489
Genotype × Herbivory	27, 331	1.17	0.257	27, 331	1.55	**0.042**	27, 330	1.62	**0.027**

Significant *P* values ($P < 0.05$) are in bold. PC1 is a late fitness component, PC2 is an early fitness component and PC3 is an indicator of flowering time (see text for details).

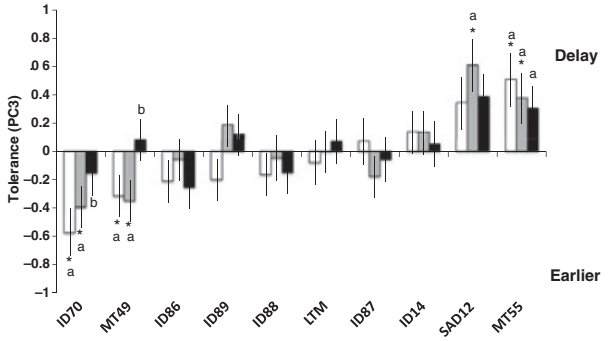

Fig. 1 Variation in tolerance, estimated as the average (adjusted LS-MEANS ± 1SE) difference in fitness (fitness component PC3, correlated with flowering time) between the damaged treatments and the undamaged controls (the zero line in the graph), for three types of herbivore damage among 10 different genotypes of *Boechera stricta*. Significant differences ($P < 0.05$) from undamaged control plants are depicted with an asterisk based on tests of simple main effects (see Materials and Methods section for details). For each genotype, the letters above the bars not identified by the same letter are significantly different ($P < 0.05$). Treatments: open bars, *Pieris rapae* (specialist); gray bars, *Trichoplusia ni* (generalist); black bars, clipped.

Table 3 Tests of simple main effects (interaction slices) for the effect of herbivore treatment on fitness components correlated with early fitness/flower number (PC2) and flowering time (PC3) for each *Boechera stricta* genotype

			PC2		PC3	
Effect	Genotype	df	*F*	*P*	*F*	*P*
Herbivory	ID14-76A	3, 330	2.90	**0.035**	0.26	0.85
Herbivory	ID70-10A	3, 330	3.88	**0.009**	3.57	**0.014**
Herbivory	ID86-38A	3, 330	0.09	0.96	0.99	0.39
Herbivory	ID87-31A	3, 330	0.58	0.62	0.66	0.58
Herbivory	ID88-2A	3, 330	1.55	0.20	0.41	0.75
Herbivory	ID89-5A	3, 330	0.43	0.73	1.91	0.13
Herbivory	LTM	3, 330	0.23	0.87	0.21	0.89
Herbivory	MT49-18B	3, 330	0.48	0.69	3.19	**0.023**
Herbivory	MT55-9B	3, 330	5.57	< **0.0001**	2.17	0.09*
Herbivory	SAD12	3, 330	0.28	0.84	2.54	**0.05**

Significant values ($P < 0.05$) are in bold.
*, Interaction with the 'specialist' and 'generalist' levels is significant at $P < 0.05$. Also see Fig. 1.

10) were able to compensate for herbivore damage, but three of 10 did not (Table 3, Fig. 2). Thus, herbivore damage to the ID14, ID70 and MT55 genotypes had a negative impact on fitness (Fig. 2), although the magnitude and impact of each type of herbivore damage also varied among these genotypes (Fig. 2). For the remaining genotypes, the type of damage (i.e. treatment) had no detectable effect on PC2, an indicator of early fecundity and flower number.

MT55 damaged plants had lower PC2 fitness compared with undamaged plants regardless of the type of damage (Fig. 2). For the ID70 genotype, only plants subjected to the specialist treatment had significantly reduced fitness relative to undamaged controls. Furthermore, for the ID14 genotype, plants in both the insect specialist and generalist treatments had significant lower fitness than undamaged control plants. Finally, plant size had a significant positive effect on fitness component PC2 ($F = 31.96$, df = 1, 323, $P < 0.0001$; 0.022 ± 0.004, estimate ± 1SE value).

The late fitness component PC1 (which is correlated with seed production) was affected significantly by genotype and marginally by herbivore treatment (Table 2). Thus, plants in the manual clipping treatment had lower fitness (PC1 LSMEANS ± 1SE, −0.16 ± 0.11) than undamaged control plants (0.07 ± 0.11; contrast test: $F_{1,331} = 3$, 59, $P = 0.059$) or plants subjected to the insect specialist treatment (0.17 ± 0.12; contrast test: $F_{1,332} = 6$, 62, $P = 0.01$), but not significantly different from plants in the generalist treatment (0.013 ± 0.11; contrast test: $F_{1,331} = 1$, 9, $P = 0.16$). Among genotypes, PC1 values ranged between 0.57 ± 0.18 for genotype MT55 to −0.77 ± 0.16 for genotype ID70. The genotype by herbivore interaction was not significant (Table 2), indicating that the effect of the type of herbivore damage on the late fitness component PC1 was constant for all genotypes. Further, this nonsignificant interaction term also indicates that there is no detectable genetic variation in tolerance to damage when late fitness components are considered.

Effect of glucosinolate polymorphism on tolerance to damage and plant fecundity

The type of glucosinolate profile was unrelated to tolerance to damage for any of the fitness components or types of damage considered (see Table S4). However, variation in

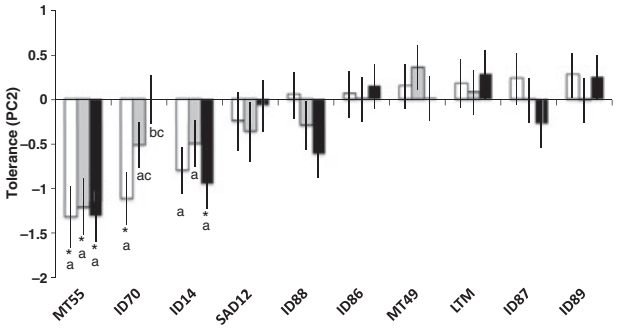

Fig. 2 Variation in tolerance, estimated as the average (adjusted LS-MEANS ± 1SE) difference in fitness (early fitness component PC2) between the damaged treatments and the undamaged controls (the zero line in the graph), for three types of herbivore damage among 10 different genotypes of *Boechera stricta*. Significant differences ($P < 0.05$) from undamaged control plants are depicted with an asterisk based on tests of simple main effects (see Materials and Methods section for details). For each genotype, letters above the bars not identified by the same letter are significantly different ($P < 0.05$). Treatments: open bars, *Pieris rapae* (specialist); gray bars, *Trichoplusia ni* (generalist); black bars, clipped.

the constitutive glucosinolate profile was associated with differences in all plant fitness components, irrespective of herbivore treatment, as indicated by the nonsignificant interaction between glucosinolate type and herbivore treatment (Table 4). Thus, plants with high proportions of BCAA-derived glucosinolates had significantly lower fitness than plants with methionine-derived glucosinolates (Fig. 3). Similarly, plants with a high proportion of BCAA-derived glucosinolates flowered later than plants with methionine-derived glucosinolates (Fig. 3). Finally, there was a significant positive effect of plant size on fitness components PC2 and PC3 (0.022 ± 0.0031, 0.0027 ± 0.004; estimate ± 1SE values for PC2 and PC3 respectively, see Table 4).

Allocation costs of tolerance

Because significant genetic variation was only detected for the PC2 and PC3 fitness components, we analyzed the costs of tolerance only for these traits. PC2 genetic means on damaged and undamaged plants were not significantly correlated (Fig. 4). However, genotype PC3 fitness means for damaged and undamaged plants showed a significant positive correlation (Fig. 4), which suggests that genotypes with late flowering times in the undamaged treatment also showed late times to flower in all the damage treatments (Fig. 4).

For both fitness components PC2 and PC3, genotypic mean tolerance to damage in each of the treatments was positive and significantly correlated in most cases (Fig. 5). This suggests that plants of a given genotype show similar levels of tolerance to different herbivore species.

Trade-offs between tolerance and chemical defenses

The constitutive concentration of leaf glucosinolates varied significantly among the 10 genotypes (ANOVA result for genotype factor: $F_{9,82} = 10.61$, $P < 0.0001$), and also between plants with different chemical profiles (ANOVA: $F_{1,90} = 5.33$, $P = 0.023$; Fig. S4). However, overall, we did not detect a significant genetic correlation between the total concentration of constitutive glucosinolates and tolerance to damage (Table 5). We detected a significant effect, dependent on the glucosinolate group, of the constitutive glucosinolate concentration on tolerance (PC2) to clipping (Table 5). Tolerance and defensive metabolite concentrations were significantly and positively genetically correlated in plants with methionine-derived glucosinolates (pairwise correlation coefficient: $n = 6$, $r = 0.93$, $P = 0.007$; Fig. S5), but not in plants with BCAA-derived glucosinolates ($n = 4$, $r = 0.54$, $P = 0.45$). These results provide no evidence for a trade-off between tolerance and resistance for each type of damage.

The total concentration of leaf constitutive glucosinolates after 24 h of damage varied significantly among genotypes and herbivore treatments (Figs 6, S6, Table S5), but genetic variation in glucosinolate induction was nonsignificant (the interaction of genotype and treatment was not significant; Table S5). The induction of glucosinolates was significantly

Table 4 Results of general linear mixed models analyzing the effect of the glucosinolate profile and herbivore treatment on three plant fitness components of *Boechera stricta*

	Fitness components								
	PC1			PC2			PC3		
Source	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Herbivory	3, 363	2.40	0.070	3, 363	1.58	0.19	3, 362	0.48	0.69
Glucosinolate profile (GS)	1, 363	15.28	**< 0.0001**	1, 363	8.47	**0.004**	1, 362	248.23	< **0.0001**
Herbivory × GS	3, 363	1.69	0.17	3, 363	1.03	0.38	3, 362	0.23	0.88
Covariate									
Plant size	1, 349	1.35	0.25	1, 348	49.27	< **0.0001**	1, 369	49.75	< **0.0001**

Significant *P* values ($P < 0.05$) are in bold. PC1 is a late fitness component, PC2 is an early fitness component and PC3 depicts flowering time. See text for details.

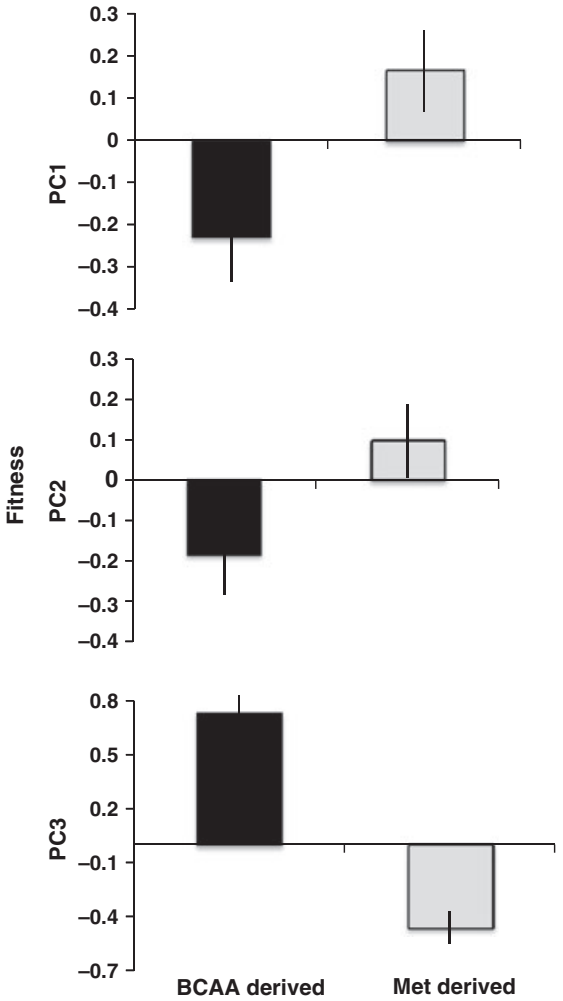

Fig. 3 Variation in plant fitness components among *Boechera stricta* plants with contrasting structural glucosinolate profiles: high proportion of branched-chain amino acid (BCAA)-derived glucosinolates (dark gray bars) vs low proportion of BCAA-derived glucosinolates (light gray bars). Met, methionine. PC1 is an indicator of late fitness/seed production, PC2 is correlated with flowering number/early fitness and PC3 is associated with variation in flowering time. Values are adjusted LS-MEANS ± 1SE.

related to the genotypic chemical profile in the specialist herbivore treatment only (Kruskall–Wallis ANOVA: df = 1, 10, χ^2 = 3.68, P = 0.05; $P > 0.05$ for the rest of the treatments; see also Fig. S7). Overall, glucosinolate induction did not affect tolerance to herbivore damage (Table S6). Only in one case did glucosinolate induction significantly affect tolerance (PC3 component) to specialist damage (Table S6), but this correlation was positive (pairwise correlation: n = 10, r = 0.65, P = 0.043).

Discussion

Our experiments show significant genetic variation for tolerance to different types of herbivore damage among genotypes of *B. stricta*, a wild relative of *Arabidopsis*. We found genetic heterogeneity in flowering responses to damage by different herbivore treatments. Different types of damage had heterogeneous effects on flowering time (PC3) and the number of flowers (PC2), and these responses varied among genotypes. By contrast, the influence of damage treatments on later fitness components (PC1) was genetically homogeneous. Variation in the structural glucosinolate profile did not influence tolerance to damage, although it is associated with aspects of plant fitness. Plants with a high proportion of BCAA-derived foliar glucosinolates had significantly later flowering times and lower fitness than plants containing methionine-derived glucosinolates. We did not find a significant negative genetic correlation between tolerance and fitness in the absence of herbivores, or between tolerance to different types of herbivore damage. Finally, we did not detect any trade-off between tolerance and resistance that might explain the genetic variation in tolerance among these genotypes.

Although this study does not include native herbivores of *B. stricta*, results from our experiment are relevant because we analyzed the effect on tolerance to damage by different types of herbivory, and also examined genotypes with contrasting constitutive chemical background, which are understudied aspects in the context of plant tolerance to herbivore damage.

Genetic variation for tolerance and effects of types of herbivory on plant fitness

Effects of herbivory on plant fitness range from mortality to overcompensation (reviewed by Agrawal, 2000b; Hawkes & Sullivan, 2001; Wise & Abrahamson, 2007). Within host species, responses to herbivory are determined by the degree of genetic variation, environmental effects and their complex interactions (Strauss & Agrawal, 1999; Fornoni *et al.*, 2003; Nuñez-Farfán *et al.*, 2007). Our results support this view. Leaf herbivory affected fitness-related traits in *B. stricta*, although both the magnitude and direction of these effects varied among genotypes, as well as among different fitness components and types of herbivore damage. Despite progress in this field, analyses of tolerance in response to different insects and modes of herbivory are still scarce (Agrawal *et al.*, 1999; Jones *et al.*, 2006).

The type of herbivory and the distribution of damage within the plant are believed to influence tolerance and its expression across genotypes (Rosenthal & Kotanen, 1994; Agrawal *et al.*, 1999). This view is grounded in two well-known facts: (1) different herbivores feed on different plant tissues and structures, which may affect plant performance differently according to the fitness value of the tissue (e.g. Karban & Strauss, 1993; Zangerl & Rutledge, 1996; Anderson & Agrell, 2005; Barto & Cipollini, 2005); (2) induced resistance in plants is herbivore specific, as are the

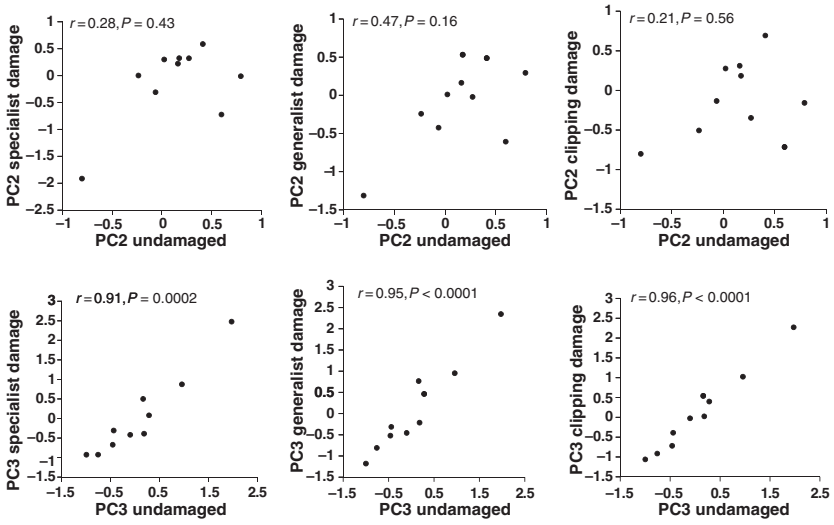

Fig. 4 Genetic correlations between mean fitness components correlated with early fecundity PC2 (above) and flowering time PC3 (below) between damaged *Boechera stricta* plants and their undamaged controls. Values in the figure are the genetic correlation coefficients. A significant negative correlation between the fitness of damaged and undamaged plants would indicate a cost of tolerance. However, no evidence for such costs is detectable.

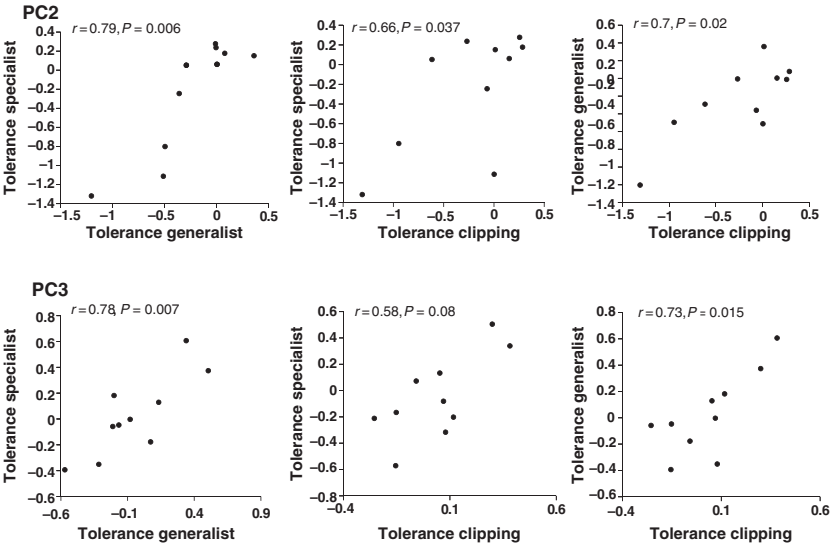

Fig. 5 Genetic correlations for tolerance to different damage treatments. For each *Boechera stricta* genotype (each point in the graphs), tolerance is estimated as the average (adjusted LS-MEANS) difference in fitness between the damaged treatments and the undamaged controls. Each panel indicates the genetic correlation and its statistical significance. The early fitness component PC2 is in the top row, and the flowering time fitness component PC3 is below. A significant negative correlation between the tolerance of different types of damage would indicate a cost of tolerance. However, no evidence for such costs is detectable.

consequences for plant fitness (Agrawal, 1998, 1999, 2000a). In our experiment, the amount of damage imposed was constant across plant genotypes and herbivore treatments, yet the distribution of this damage within the plant differed among treatments. *Pieris rapae* larvae fed mostly on the upper parts of the plant (primarily on young and recently mature upper leaves), whereas *T. ni* larvae fed on lower leaves and avoided feeding on the apical part of the plant (Fig. S3). By contrast, in the clipping treatment, the damage was homogeneously distributed within the plant (see the Materials and Methods section). In addition, we found that induced resistance varied significantly among herbivore treatments, and this variation was homogeneous among genotypes (Figs 6, S6 and Table S5). In particular, we found that induced glucosinolate responses were higher in plants attacked by *P. rapae* larvae than in other herbivore treatments. However, although the herbivore treatments differed consistently across genotypes in terms of the location of the damage and the pattern of induced response, the effect of the type of damage on tolerance was heterogeneous among genotypes (Table 2). MANOVA detected significant genetic variation on overall fitness traits, with significant responses to herbivory at earlier stages of plant reproduction (i.e. on flowering time and the total number of flowers). Two of the genotypes flowered earlier when exposed to insect damage, and insect herbivory caused two other genotypes to delay flowering in comparison with their undamaged controls (Fig. 1). Similarly, herbivory had a significant impact on the total number of flowers produced per plant, yet the magnitude of these effects were genotype specific and dependent on the type of damage. When significant (in three of 10 genotypes), herbivory always had a

Table 5 Results of the robust ANCOVA analyzing trade-offs between constitutive resistance (genotype mean glucosinolate concentration, GS_0) and tolerance to three types of herbivore damage across 10 *Boechera stricta* genotypes with two contrasting constitutive glucosinolate profiles (BCAA-derived glucosinolates vs methionine-derived glucosinolates)

		Tolerance PC2						Tolerance PC3					
		Specialist		Generalist		Clipping		Specialist		Generalist		Clipping	
Effect	df	R_n^2	*P*	R_n^2	*P*	R_n^2	*P*	R_n^2	*P*	R_n^2	*P*	R_n^2	*P*
$[GS_0]$	1, 10	0.21	0.64	0.365	0.544	2.84	0.09	0.275	0.6	0.291	0.589	0.06	0.8
GS profile	1, 10	0.01	0.91	0.013	0.907	33.61	**< 0.0001**	1.181	0.177	2.161	0.141	1.17	0.278
GS profile × $[GS_0]$	1, 10	0.04	0.83	0.045	0.831	23.83	**< 0.0001**	1.31	0.252	0.937	0.332	0.547	0.459

Significant *P* values ($P < 0.05$) are in bold.

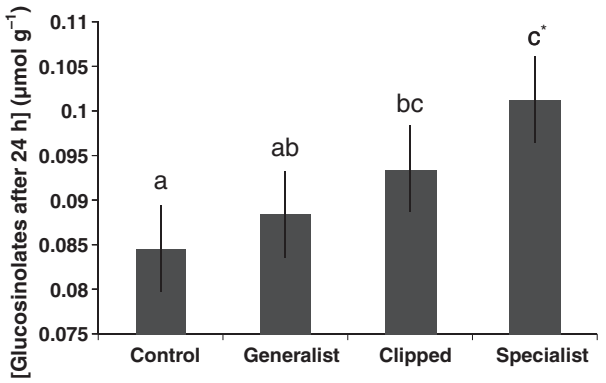

Fig. 6 Total *Boechera stricta* leaf glucosinolate concentration (adjusted LS-MEANS ± 1SE) after 24 h of damage among four different herbivore treatments. Different letters depict significant differences ($P < 0.05$). *, Marginally significant ($P = 0.07$).

negative effect, resulting in fewer flowers than in the undamaged controls.

The marginally significant effect of the type of damage on later fitness components (PC1) is also noticeable, which suggests that plants damaged by the specialist insect tended to overcompensate, whereas clipped plants tended to undercompensate, and this was constant for all the genotypes (as indicated by the nonsignificance of the herbivory by genotype interaction for the component PC1, see Table 2). Overcompensation following *P. rapae* damage has been shown previously in the Brassicaceae family, is related to the induced response to such damage, and is thought to be adaptive (i.e. plants have enhanced fitness when induced by specialist insects, and lower fitness when undamaged or clipped; see Agrawal, 1998, 1999). Similarly, overcompensation is common when plants are exposed to apical damage (Wise & Abrahamson, 2008, and references therein), which was the main type of damage inflicted by *P. rapae* larvae in our experiment.

Our results show that, although *B. stricta* can compensate for herbivory (particularly damage caused by the specialist insect), herbivory still reduces overall flower production and alters flowering for some genotypes. The mechanisms involved in compensation for herbivory are beyond the scope of this paper, but differential allocation of resources to other plant tissues, variation in flowering time and changes in growth rates are known mechanisms (Rosenthal & Kotanen, 1994; Agrawal *et al.*, 1999; see also Pilson & Decker, 2002) which might explain the observed variation in tolerance to herbivory among *B. stricta* genotypes.

Clearly, the expression of tolerance may be influenced by other ecological factors not considered in our study. For example, the levels of interspecific competition experienced in the field (Jones *et al.*, 2006) and the timing of damage may be important factors determining the tolerance of *B. stricta* to herbivory. We damaged the plants after 6 wk of vegetative growth, which may correspond to the mid-summer period when most herbivores are actively feeding on *B. stricta* plants in natural populations (T. Mitchell-Olds, pers. obs.). However, later herbivory is still possible if a herbivore outbreak occurs late in the season, or if plant phenology is altered by local climatic conditions. The later in the season that the damage occurs, the shorter the compensation period will be, and hence damage may also affect late fecundity (see Maschinski & Whitham, 1989; Tiffin, 2002).

Glucosinolate polymorphism and plant fecundity

A first step to understanding the significance underlying the polymorphism of plant chemical defenses is the determination of whether such variation has ecological implications. Previously, in our system, we found that chemical polymorphism affected plant resistance to generalist herbivores (Schranz *et al.*, 2009). By contrast, we have shown here that tolerance to herbivore damage does not depend on the structural glucosinolate profile. In the current study, the chemical polymorphism was associated with plant fitness regardless of herbivore damage. Thus, plants with a high proportion of BCAA-derived glucosinolates in their leaves flowered significantly later and had significantly lower fitness than plants containing mainly methionine-derived

glucosinolates (Fig. 3). Previous studies have shown negative effects of chemical defenses on plant fecundity in several systems, suggesting the existence of direct allocation costs of resistance (Strauss *et al.*, 2002). However, to our knowledge, no work has described the existence of direct costs of resistance derived from natural and discrete variation in the structural chemical profile within plant species. Although this effect on fitness might also reflect other genotypic effects (i.e. linkage disequilibrium), several arguments suggest that discrete variation in the chemical profile of *B. stricta* genotypes might truly affect other components of plant fitness. First, among plant species, the magnitude and significance of constitutive resistance costs at the quantitative level vary among types of defensive compounds, because distinct defensive compounds have different biosynthetic costs (Koricheva, 2002 and references therein). Interestingly, in our system, we also found a significant negative genetic correlation between the total concentration of glucosinolates and fitness components among genotypes with different chemical profiles, suggesting a direct cost of resistance. Although the total concentration of glucosinolates was negatively correlated with early plant fecundity (PC2), it was strongly and negatively correlated with late plant fecundity (PC1) in genotypes with a high proportion of BCAA-derived glucosinolates (Table S7, Fig. S8).

Second, QTL mapping analyses recently conducted on a *B. stricta* mapping population have revealed a significant QTL predicting survival and reproduction in the *BCMA* chromosomal region, which controls the synthesis of BCAA- vs methionine-derived glucosinolates in *B. stricta* (Schranz *et al.*, 2009; J. Anderson & T. Mitchell-Olds, unpublished). Future work using transgenic lines will clarify whether *BCMA* genes have significant effects on plant fitness traits.

One limitation of our study, however, is that fitness estimates were obtained under glasshouse conditions, which may differ from fitness measurements in natural populations, especially if variation in the chemical profile in *B. stricta* is the result of local adaptation or another form of balancing selection. The latter possibility is currently being examined in natural populations of *B. stricta*.

Costs of tolerance

Plant defense theory proposes that allocation costs maintain genetic variation for tolerance, preventing the fixation of alleles for maximal tolerance among individuals within and among populations (Strauss & Agrawal, 1999; Strauss *et al.*, 2002). However, empirical evidence for such costs is limited, despite multiple attempts to address this question (reviewed by Koricheva, 2002; Leimu & Koricheva, 2006). Here, we investigated two different types of allocation costs of tolerance. However, we did not find a significant negative genetic correlation between tolerance and fitness in the absence of herbivores, or between tolerance to different types of herbivore damage. Indeed, tolerance to different types of damage showed significant positive genetic correlations. These results indicate that allocation costs do not constrain the evolution of tolerance (hence genetic variation for tolerance may be maintained by other ecological or evolutionary forces) and genotypes showed positive genetic correlations in their tolerance to different herbivore treatments. This result is concordant with the few existing studies examining tolerance to different enemies, which found no evidence for trade-offs between tolerance to different types of damage (Tiffin & Rausher, 1999; Pilson, 2000). These results suggest that the evolution of tolerance to multiple herbivores may not account for the existence of intermediate levels of tolerance to leaf damage on plants (Nuñez-Farfán *et al.*, 2007).

In addition, we did not find any trade-off between tolerance and investment in defensive metabolites, as suggested by the lack of a significant negative genetic relationship between constitutive or induced glucosinolates and tolerance to different types of damage. On the contrary, we found that the concentration of constitutive glucosinolates and tolerance to clipping were significantly and positively correlated, at least in terms of the production of flowers. This suggests that tolerant plants may also be more resistant to some types of damage. This was especially true for genotypes with methionine-derived glucosinolates (Table 5, Fig. S5). Thus, the evolution of resistance and tolerance in *B. stricta* does not seem to be limited by genetic constraints and, for genotypes with methionine-derived glucosinolates, resistance and tolerance will evolve jointly (i.e. the allocation of resources simultaneously to both tolerance and resistance; Nuñez-Farfán *et al.*, 2007).

Although we did not detect any allocation costs of tolerance in our study, there is growing evidence that the existence of allocation costs is dependent on complex interactions with the biotic and/or abiotic environment (Koricheva, 2002; Siemens *et al.*, 2009), which we did not manipulate in our glasshouse experiment. Therefore, future work will need to take into account the environmental variation that exists under natural conditions.

In short, our study has shown that structural variations in the chemical profile of plant defenses do not influence the ability of *B. stricta* to compensate for different types of herbivore damage, although this chemical variation is correlated with plant fitness in our sample. In addition, our study demonstrates the importance of considering jointly both intrinsic plant factors and extrinsic ecological factors to understand the evolution of plant tolerance to damage and its costs.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Distribution map of *Boechera stricta* populations and geographical origin of the genotypes selected for the tolerance experiment.

Fig. S2 Schematic representation of *Boechera stricta* showing the three different ontogenetic stages of leaf growth.

Fig. S3 Average percentage of leaves harmed and average damage per leaf caused by the herbivores among three different ontogenetic levels within 215 *Boechera stricta* plants used in the insect treatments in the tolerance experiment.

Fig. S4 Variation in the total leaf glucosinolate concentration among 90 *Boechera stricta* plants with two contrasting structural glucosinolate profiles.

Fig. S5 Genetic correlations between the early fecundity fitness component and the average total concentration of leaf glucosinolates of *Boechera stricta* plants with methionine-derived glucosinolates.

Fig. S6 Genetic correlations between the mean total leaf glucosinolate concentration of undamaged control plants and the mean total leaf glucosinolate concentration after 24 h of damage treatment to three types of herbivory.

Fig. S7 Variation in the total leaf glucosinolate concentration after 24 h of damage caused by the specialist lepidopteran *Pieris rapae* among *Boechera stricta* plants with two contrasting structural glucosinolate profiles: plants with a high proportion of branched-chain amino acid (BCAA)-derived glucosinolates vs plants with methionine-derived glucosinolates.

Fig. S8 Genetic correlations between the average total concentration of leaf glucosinolates and the late fitness component, shown separately for genotypes with predominantly branched-chain amino acid (BCAA)- and methionine-derived glucosinolates.

Notes S1 Insect feeding behavior and intraplant variation in plant damage.

Notes S2 Constitutive and induced glucosinolate analysis and methods.

Table S1 Location and geographical origin of the 10 genotypes used in the tolerance to damage experiment

Table S2 Pairwise correlation coefficients among the four fitness components considered in this study

Table S3 Principal component analysis conducted on four plant reproductive parameters of 368 *Boechera stricta* plants under four different herbivore treatments

Table S4 Results of Kruskall–Wallis tests conducted to analyze the differences in tolerance to three types of herbivore damage between *Boechera stricta* plants with two contrasting glucosinolate profiles

Table S5 Summary results of the general linear mixed model testing the effects of herbivore treatment, genotype and their interaction on the total leaf glucosinolate concentration after 24 h of herbivore damage

Table S6 Results of the robust ANCOVA analyzing the trade-offs between induced resistance and tolerance to three types of herbivore damage across 10 *Boechera*

stricta genotypes with two contrasting constitutive glucosinolate types

Table S7 Results of the robust ANCOVA analyzing the direct cost of resistance for *Boechera stricta* plants with two contrasting constitutive glucosinolate types

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