Title:

Plasticity of plant defense and its evolutionary implications in wild populations of *Boechera stricta*

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Abstract:

Phenotypic plasticity is thought to impact evolutionary trajectories by shifting trait values in a direction that is either favored by natural selection ("adaptive plasticity") or disfavored ("nonadaptive" plasticity). However, it is unclear how commonly each of these types of plasticity occurs in natural populations. To answer this question, we measured glucosinolate defensive chemistry and reproductive fitness in over 1,500 individuals of the wild perennial mustard *Boechera stricta*, planted in four common gardens across central Idaho, USA. Glucosinolate profiles—including total glucosinolate quantity as well as the relative abundances and overall diversity of different compounds—were strongly plastic both among habitats and within habitats. Patterns of glucosinolate plasticity varied greatly among genotypes. More often than expected by chance, glucosinolate profiles shifted in a direction that matched the direction of natural selection, indicating that plasticity among habitats tended to increase relative fitness. In contrast, we found no evidence for within-habitat selection on glucosinolate reaction norm slopes (i.e., plasticity along a continuous environmental gradient). Together, our results indicate that glucosinolate plasticity may improve the ability of *B. stricta* populations to persist after migration to new habitats.

Introduction

The role of phenotypic plasticity in adaptive evolution has been a subject of great controversy and research interest for decades (Bradshaw 1965; Via and Lande 1985; Via *et al.* 1995; Pigliucci 2005; Ghalambor *et al.* 2015; Hendry 2015). It has long been recognized that both an organism's genotype and its environment shape its phenotype, which then determines its evolutionary fitness. Strictly speaking, phenotypic variation caused by environmental stimuli is not heritable and therefore cannot result in evolution through systematic changes in allele frequencies (Falconer and Mackay 1996). Nevertheless, plasticity is predicted to impact evolution by shifting phenotypes that are under natural selection (Bradshaw 1965). Furthermore, if patterns of plasticity are genetically variable, then plasticity itself may evolve in response to selection (Gomulkiewicz and Kirkpatrick 1992). It remains unclear how commonly these phenomena occur in natural populations, and whether the adaptive value of plasticity varies for different traits, environments, and spatial scales.

One way that plasticity could impact evolution is by accelerating or hindering adaptation to a novel environment—e.g., upon invasion of a new habitat or in response to a relatively sudden ecosystem shift, as might result from climate change (Donohue *et al.* 2001; Richards *et al.* 2006; Ghalambor *et al.* 2007; Anderson *et al.* 2012). Plasticity that moves a phenotype closer to the new phenotypic optimum is often called "adaptive" plasticity because it increases fitness relative to a non-plastic genotype (Figure 1g-i); however, whether this type of plasticity actually facilitates genetic adaptation is controversial. Strong

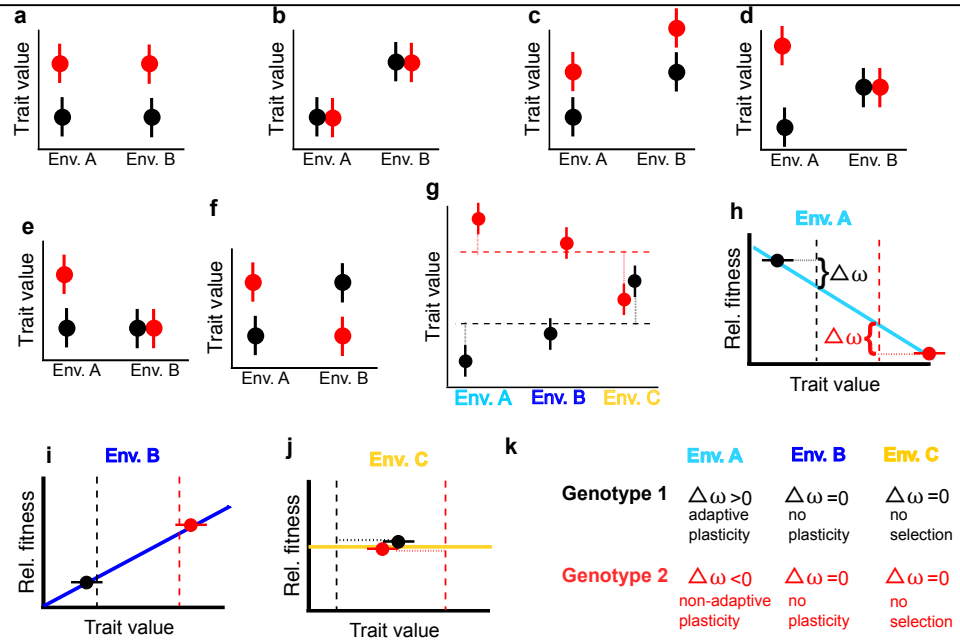

Figure 1: Schematic of genotype-by-environment interactions and adaptive plasticity between discrete environments. Points show mean trait values of two genotypes ("red" and "black"). Panel **(a)** depicts a trait that is under pure genetic control with no plasticity. Panel **(b)** depicts a trait that is plastic but not genetically variable. Panel **(c)** shows both a genotype effect and plasticity, but no interaction between them. Panels **(d)-(f)** depict examples of genotype-by-environment interactions. In **(d)**, a genetic difference is detectable only in one site; the genotypes have plasticity of equal magnitude but opposite sign so that the mean phenotype in each site is identical. In **(e)**, only one genotype is plastic. In **(f)**, the genotypes switch rank phenotype; averaged across sites, there is no genetic difference between them, and the average trait value within each site is the same. Note that panels **(a)-(f)** could represent six different traits measured simultaneously in one experiment: plasticity is a property of a particular trait, a particular genotype, and a particular environmental change. Panels **(g)-(k)** illustrate how "adaptive" and "nonadaptive" plasticity among environments can be detected. First, each genotype's trait value in each site can be compared to its average trait value across environments (depicted as dashed red or black lines, horizontal in **g** and vertical in **h-j**, representing a hypothetical genotype that is identical except that it is completely nonplastic). Next, a selection differential (an equation expressing relative fitness as a function of trait values, shown as solid colored lines in **h-j**) is calculated for each environment. To determine the expected change in fitness due to plasticity, the selection differential is evaluated at the "no-plasticity" baseline trait value (dashed lines) and at the genotype's true trait value observed in that environment. **(k)** In hypothetical Environment A, one genotype shows adaptive plasticity and the other shows nonadaptive plasticity. In Environment B, neither genotype shows a significant plastic deviation from its experiment-wide mean. In Environment C, both genotypes show substantial plastic deviations, but the trait is not under selection so the plasticity has no effect on fitness.

adaptive plasticity could place an organism very near to the new adaptive peak, removing the selective force that would otherwise drive adaptation through allele frequency change and thereby inhibiting local adaptation. Alternatively, moderate adaptive plasticity may enable a population to survive in the new environment long enough for selection to increase the frequency of beneficial alleles, thus promoting local adaptation (Baldwin 1896; Price *et al.* 2003; Ghalambor *et al.* 2007). The opposite pattern—in which "nonadaptive" plasticity moves phenotypes farther from the new optimum—may either increase the risk of extinction or lead to rapid adaptive evolution by intensifying natural selection (Conover and Schultz 1995; Ghalambor *et al.* 2007; Ghalambor *et al.* 2015; Huang and Agrawal 2016). In this manuscript, we do not attempt to determine whether plasticity constrains or facilitates long-term genetic adaptation. Rather, our goal is to assess the relative frequency of adaptive versus nonadaptive plasticity in natural populations.

A related but distinct question is whether, and how, plasticity might evolve as an adaptation to environmental heterogeneity within a single habitat. Plasticity in response to fine-scale environmental variation is often imagined as a *reaction norm*, with the trait value as some function of a continuous environmental predictor (Figure 2; Schmalhausen 1949). For traits that exhibit genotype-by-environment interactions, genetic variation exists for reaction norm shape. Natural selection acting on variation for plasticity can be detected using established quantitative genetic methods such as genotypic selection analysis on reaction norm coefficients (Figure 2g-h; Weis and Gorman 1990; Rausher 1992; Baythavong and Stanton 2010). Natural selection is predicted to result in

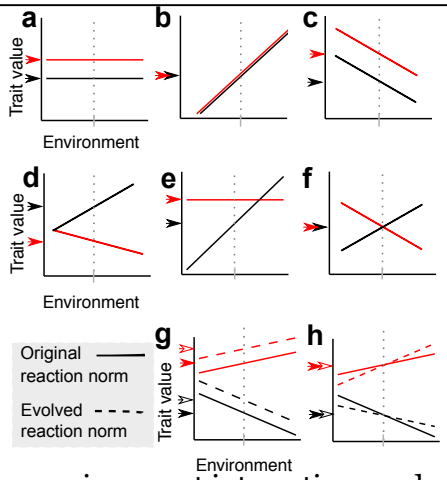

Figure 2: Genotype-environment interactions and selection on plasticity over continuous environmental gradients: Examples of reaction norms. Lines show the mean trait values of two genotypes ("red" and "black") across a range of some continuous environmental predictor. The arrows on the vertical axis indicate reaction norm *height*, or the trait value for each genotype evaluated at the average value of the environmental predictor (indicated by grey tick marks on horizontal axis and vertical dotted lines). Panel **(a)** shows a genetic difference with no plasticity—i.e., zero slope. Panel **(b)** shows plasticity with no genetic difference. Panel **(c)** shows both plasticity and a genotype effect, but no interaction between them, indicated by parallel reaction norms—the genotypes differ in reaction norm height, but not slope. Panels **(d)-(f)** all show possible genotype-by-environment interactions, or genetic variation for reaction norm shape. In panel **(d)** reaction norm slopes differ in sign but not magnitude; in **(e)** only one genotype is plastic. In panel **(f)** the genotypes are indistinguishable when averaged across all environments (e.g., if the environmental gradient was unobserved); the genotype difference is environment-dependent. When mean trait values and plasticity are genetically uncorrelated, they can evolve independently. Panel **(g)** illustrates changes in reaction norm height in response to linear selection for increased mean trait values. Panel **(h)** illustrates evolutionary change in reaction norm slope in response to linear selection for more positive linear reaction norm coefficients. Note that such selection may result in either increased or decreased overall plasticity (steeper or shallower slope), depending on the original shape of the reaction norm.

increased plasticity if patterns of plasticity are heritable, if the spatial scale of changing selective pressures is similar to the organism's dispersal distance (Levins 1962; Gomulkiewicz and Kirkpatrick 1992; Baythavong 2011), if reliable environmental cues for the selection pressure are available (Levins 1963; Donohue *et al.* 2000; Schmitt *et al.* 2003; Reed *et al.* 2010), and if costs of plasticity are minimal (Auld *et al.* 2010).

62 Despite several excellent empirical studies (Dudley and Schmitt 1996;
63 Schmitt *et al.* 1999; Donohue *et al.* 2000; Donohue *et al.* 2001; Sultan 2001;
64 Baythavong 2011), more examples from natural populations are needed to test
65 theoretical predictions about the fitness impacts of both between-environment
66 and within-environment plasticity (Hendry 2015). Data on the plasticity and
67 evolution of physiological traits (as opposed to morphological or life-history
68 traits) is particularly scarce (Palacio-López *et al.* 2015). Because variation in
69 phytochemistry may affect not only the evolution of the plant but also entire
70 communities and ecosystems (Wimp *et al.* 2007; Hopkins *et al.* 2009),
71 phytochemical plasticity has been identified as a high priority research target
72 (Hendry 2015). Here, we address these needs by studying plasticity and evolution
73 of glucosinolate defensive chemistry in the wild perennial herb *Boechera stricta*,
74 a close relative of Arabidopsis. Goals of this study were (1) to characterize
75 genotype-by-environment interactions underlying glucosinolate variation in *B.*
76 *stricta*, (2) to assess whether glucosinolate plasticity alters relative fitness after
77 transition to novel habitats, and (3) to test whether natural selection acts on
78 glucosinolate reaction norms within habitats.
79 We measured glucosinolate profiles, size, and fecundity of 25 *B. stricta*
80 genotypes replicated in 80 experimental blocks divided among four common
81 gardens in diverse habitats (Figure 3). Because *Boechera* has limited dispersal
82 (<0.5 m on average; Bloom *et al.* 2002), the environmental variation
83 encompassed by the widely separated common gardens is much greater than
84 what individual *B. stricta* populations normally encounter; thus, plasticity among
85 field sites describes plasticity after a sudden environmental change or migration

to a new habitat. To assess whether glucosinolate plasticity among habitats exhibits an “adaptive” or “nonadaptive” pattern, we compared the direction of selection in each site with the direction of diverse genotypes’ plastic responses to that site (Figure 1g-k). Then, we quantified within-habitat glucosinolate plasticity and assessed its relationship to fecundity in each habitat using genotypic selection analysis on reaction norm coefficients (Figure 2g-h). We found that substantial genotype-by-environment interactions underlie glucosinolate variation in *B. stricta*, and plasticity among sites tended to move trait values in an adaptive direction; however, we did not detect selection on glucosinolate plasticity within habitats.

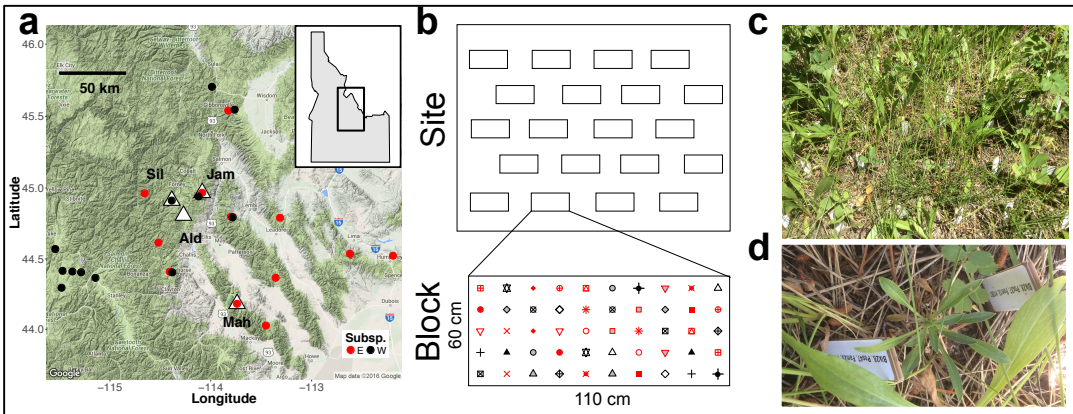

Figure 3: Field sites and experimental design. **(a)** Map of field sites and wild *B. stricta* populations used in common garden experiment. Common gardens are denoted with white triangles and labeled. Circles mark collection sites of the 25 genotypes included in the experiment. Not shown: one EASTERN genotype collected in Colorado. Map data: Google. **(b)** Each common garden contained 16 to 22 experimental blocks. Each randomized block contained two individuals of each of the 25 genotypes, planted in a 10-cm grid. Panel **(c)** shows a block placed within the natural vegetation; **(d)** shows one experimental rosette with its identifying tag.

97 # Methods

98 All statistical analyses were performed in R version 3.3.2 (R Core Team
99 2016) with heavy use of the packages ggplot2, lme4, lmerTest, dplyr, tidyr, and
100 stringr (Wickham 2009; Bates *et al.* 2015; Kuznetsova 2015; Wickham and
101 Francois 2015; Wickham 2016a,b). Throughout, *P*-values were corrected for
102 multiple comparisons using the sequential Bonferroni correction (Holm 1979).
103 Additional details for all sections are available in Supplementary Methods. All
104 data and R code will be made freely available in a Dryad repository upon
105 publication.

106 ## *Study system*

107 The short-lived perennial herb *Boechera stricta* (Graham) Al-Shehbaz is
108 common in montane meadows and forests throughout its native range in western
109 North America (Rushworth *et al.* 2011). Natural populations are strongly
110 genetically differentiated (F_{ST}=0.56; Song et al. 2006) and have adapted to
111 diverse habitats that vary in climate, water availability, elevation, soil
112 composition, plant community diversity and density, and microbial community
113 composition (Supplementary Figure 1; Anderson *et al.* 2013a,b; Wagner *et al.*
114 2016).

115 *B. stricta* produces a variety of glucosinolates, which are sulfur-rich,
116 biologically active phytochemicals that protect against generalist insect
117 herbivores and pathogens and may also affect nonpathogenic root-associated
118 microbes (Agrawal 2000; Tierens *et al.* 2001; Brader *et al.* 2006; Halkier and

119 Gershenzon 2006; Bednarek *et al.* 2009; Bressan *et al.* 2009; Hopkins *et al.*
120 2009; Schranz *et al.* 2009; Sanchez-Vallet *et al.* 2010). Glucosinolates are
121 constitutively produced, although attack by natural enemies often induces
122 additional production (Agrawal 1998, 2000; Brader *et al.* 2001; Agrawal *et al.*
123 2002; Textor and Gershenzon 2009; Abdel-Farid *et al.* 2010; Manzaneda *et al.*
124 2010). *B. stricta* produces four aliphatic glucosinolate compounds with varying
125 biological activity (Figure 4a; Windsor *et al.* 2005; Schranz *et al.* 2009; Prasad *et*
126 *al.* 2012). Total glucosinolate concentration and relative abundances of these
127 compounds vary extensively among individuals (Figure 4b-c).

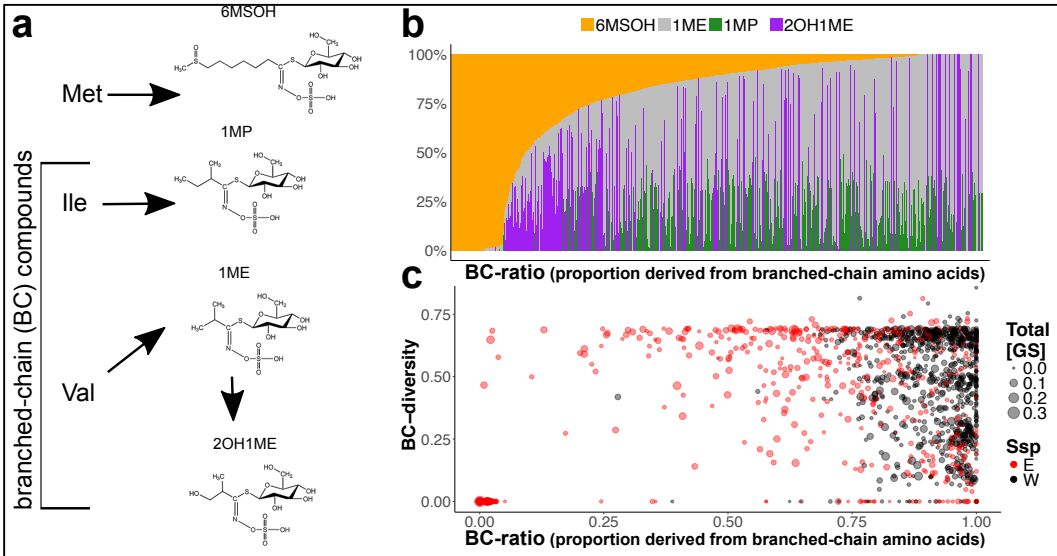

Figure 4: Glucosinolate variation in 1,505 field-grown *Boechera stricta* rosettes. **(a)** Chemical structures of the four primary glucosinolates in *B. stricta (*Kanehisa *et al.* 2002), with their amino acid precursors. 6MSOH is derived from methionine; the other compounds are all derived from branched-chain amino acids. (**b-c**) Rosette leaf glucosinolate profiles of 1,505 field-grown plants. In both panels, plants are sorted by increasing BC-ratio (i.e., decreasing 6MSOH). **(b)** The proportions of four aliphatic glucosinolates are shown for each measured plant. **(c)** Three summary metrics of each glucosinolate profile are shown for each individual. Three genotypes in this study lack branched-chain glucosinolate functionality and only produce 6MSOH; individuals of these genotypes are seen in the lower left-hand corner of panel **(c)**.

128

129 ### *Design and installation of field experiment*

130 In October 2013, we planted 4,000 self-full siblings of 25 naturally inbred
131 *Boechera stricta* genotypes (Supplementary Table 1) in fully randomized blocks
132 (two replicates per genotype per block) in four common gardens in central Idaho
133 (Figure 3). These field sites are all home to wild *B. stricta* populations, and are
134 distinguished by many biotic and abiotic environmental characteristics
135 (Supplementary Table 2; Supplementary Figure 1). Each genotype was derived
136 from an accession from one wild *B. stricta* population (Figure 3a), which we
137 propagated by self-fertilization in standard greenhouse conditions to minimize
138 variation caused by maternal environmental effects. These genotypes represent
139 the breadth of *B. stricta* genetic diversity, comprising 12 from the WEST
140 subspecies and 13 from the EAST subspecies (Lee and Mitchell-Olds 2013).
141 Because *B. stricta* primarily self-pollinates and is naturally inbred (F_{IS}=0.89;
142 Song *et al.* 2006), self-full siblings are essentially genetically identical. Therefore,
143 phenotypic variation among individuals of the same genotype describe that
144 genotype's plastic response to environmental variation.

145 ### *Measurement of plant performance in the field*

146 During summer 2014, we returned to each site several times to measure
147 survival, developmental stage, and height. At the end of the growing season, we
148 measured fruit production for each surviving individual. Because *B. stricta* is
149 predominantly self-pollinating (Song *et al.* 2006), fruit production reflects both
150 male and female fecundity, and thus is a good estimate of reproductive fitness.

151 For phenotypic selection analyses (below), we used fecundity (in mm of
152 fruit produced) as a measurement of reproductive fitness:

153 $$Fecundity = Number\ of\ siliques \times Length\ of\ average\ silique$$

154 For estimation of genotypic fitness, we also calculated the probability of
155 survival for each genotype l :

156 $$P(survival) = \frac{number\ of\ surviving\ individuals\ of genotype\ l}{number\ of\ individuals\ of\ genotype\ l\ originally\ planted}$$

157 We then calculated the total evolutionary fitness for each genotype as:

158 $$w = P(survival) \times mean(fecundity)$$

159 ***Measurement of glucosinolate profiles***

160 Because insect attack can induce additional production of glucosinolates
161 (Agrawal 1998), we measured glucosinolate profile as early as possible in the
162 summer, before peak herbivory. On the earliest census date for each site, we
163 collected ~20-30 mg of rosette leaf tissue from each surviving plant into tubes
164 containing 70% methanol. Samples were shipped to Duke University, then fully
165 randomized onto 96-well plates. Glucosinolates were extracted from the
166 methanol leachates using established protocols (Supplementary Methods). We
167 used high-performance liquid chromatography (HPLC) to measure the
168 abundance of four aliphatic glucosinolates (Figure 4a-b) in each sample. Three of
169 these compounds (1ME, 1MP, 2OH1ME) have branched-chain structures and
170 biological activity that differs from that of the fourth, straight-chain compound
171 (6MSOH; Figure 4a; Schranz *et al.* 2009; Prasad *et al.* 2012). We calculated
172 absolute concentrations (μmol per mg dry weight) of each compound by

173 comparing each peak to an internal standard and dividing by the dry weight of
174 each leaf sample (Supplementary Methods).
175 From the absolute concentrations of all four compounds, we calculated
176 three summary metrics for each sample's glucosinolate profile:

177 $$Total_{[GS]} = [2OH1ME] + [1ME] + [1MP] + [6MSOH]$$

178 $$BC_{ratio} = \frac{[2OH1ME] + [1ME] + [1MP]}{Total_{[GS]}}$$

179 $$BC_{diversity} = -\sum_{i=1}^{k} [BC_i] * \log([BC_i])$$

180 where k = the total number of branched-chain compounds present in the sample
181 and $[BC_i]$ = the concentration of the ith branched-chain glucosinolate. Total [GS]
182 describes the combined concentration of all aliphatic glucosinolates. BC-ratio
183 describes the proportion of aliphatic glucosinolates that are derived from
184 branched-chain amino acids, which is an ecologically and evolutionarily
185 important trait in *B. stricta* (Schranz *et al.* 2009; Manzaneda *et al.* 2010; Prasad
186 *et al.* 2012). Finally, BC-diversity describes the balance of the three types of
187 branched-chain glucosinolates, taking low values when glucosinolate profiles are
188 dominated by one compound and high values when multiple compounds are
189 present in similar amounts (Figure 4b-c). We calculated BC-diversity using the
190 Shannon diversity index in the **R** package **vegan** (Oksanen *et al.* 2013).
191

192 ### *Partitioning variance in glucosinolate profiles*

193 To assess plasticity of glucosinolate profiles among habitats, we used
194 univariate REML mixed models and ANCOVA to partition variance in each of the
195 three glucosinolate traits among genetic and environmental predictors. We
196 modeled each trait as:
197
198 $$Trait = Genotype + Site + Genotype * Site + Block(Site) + Genotype * Block + Plant\ height$$
199 $$+ Developmental\ stage + Batch + error$$
200
201 where *Height, Developmental Stage,* and *Batch* were nuisance variables to
202 control for (respectively) the "general vigor problem" of large plants having more
203 resources to invest in defense (Agrawal 2011), ontogenetic changes in rosette
204 glucosinolate profiles, and HPLC batch effects. *Block* (nested in *Site*),
205 *Genotype*Block*, and *Batch* were random-intercept terms; the rest were modeled
206 as fixed effects. One genotype was omitted from the analysis because no
207 individuals of that genotype survived at one field site. Spearman's rank
208 correlation tests of least-squares means resulting from this model (for the
209 Genotype fixed effect) revealed that these traits were partially genetically
210 correlated, although correlations between BC-ratio and both other traits were
211 driven by three outlier genotypes that entirely lacked branched-chain
212 glucosinolate functionality (Supplementary Table 3). Least-squares mean trait
213 values for *Site*, *Genotype*, and *Genotype* × *Site* fixed effects were used to quantify
214 plasticity among habitats.
215 To better understand how variation in individual glucosinolate compounds
216 underlies variation in these three emergent properties of glucosinolate profiles,

217 we repeated the above analysis for square-root-transformed concentrations of
218 2OH1ME, 1ME, 1MP, and 6MSOH. Similar to the emergent properties that are
219 the focus of this study, concentrations of individual compounds were strongly
220 genetically controlled but also highly plastic within and among habitats.
221 (Supplementary Tables 4, 9b; Supplementary Figures 2-3). However, genetic
222 correlations between individual compounds were even stronger than those
223 between emergent glucosinolate profile properties (Supplementary Table 3b). For
224 this reason, and because previous work has confirmed the ecological and
225 evolutionary importance of emergent glucosinolate profile properties (Prasad et
226 al. 2012; Müller et al. 2010), we focus the remainder of the analyses on BC-ratio,
227 Total [GS], and BC-diversity rather than individual compounds.

228 ***Characterizing selection on glucosinolate profiles***

229 We evaluated natural selection on three separate glucosinolate-related
230 traits (Figure 4c) in each of four common gardens (Figure 3) by conducting
231 twelve phenotypic selection analyses (3 glucosinolate traits × 4 sites). The within-
232 site relative fecundity of all measured individuals was regressed onto
233 standardized trait values, while controlling for microsite variation in habitat
234 quality using a random intercept *Block* term:
235
236 $$Individual\ relative\ fecundity = \beta * Trait_value + Block + error$$
237
238 A significant β regression coefficient indicated nonzero linear selection. This
239 regression coefficient is the selection differential on one trait at one site (*e.g.,* the
240 slopes of the lines in Figure 1h-j). The signs and magnitudes of the regression

coefficients from these models thus indicate the direction and strength of linear selection on each trait at each site.

Next, to test whether selection differentials for each trait varied among sites (i.e., whether selection was spatially variable), we analyzed the relative fecundity and standardized phenotype data from all four sites together by fitting a mixed-effects ANCOVA model with an additional *Site* × *Trait* interaction term, which described heterogeneity of linear selection on the trait among habitats:

$$Individual\ relative\ fecundity = Site + Block(Site) + Trait + Site * Trait + error$$

Because stabilizing or disruptive selection could also affect the fitness consequences of plasticity (i.e., movement of trait values either toward or away from optimum values at each site), we also conducted quadratic selection analysis for each trait at each site:

$$Individual\ relative\ fecundity = Block + \beta * Trait_{value} + \gamma * (Trait_value)^2 + error$$

A significant γ regression coefficient indicated nonzero quadratic selection; positive values were taken as evidence of stabilizing selection, while negative values indicated disruptive selection. However, quadratic regressions were included in downstream analyses only if they gave significantly improved fit over a linear selection model, and if we could reject the null hypothesis that the true fitness minimum or maximum fell outside the range of observed trait values (Mitchell-Olds and Shaw 1987).

263 ***Testing for adaptive plasticity among habitats***

264 The question of whether plasticity can aid survival in new environments
265 hinges on whether the plastic response moves trait values in a direction favored
266 by natural selection, thus increasing relative fitness in the new environment. We
267 explored this interaction between plasticity and selection by combining results
268 from the variance-partitioning model and selection analyses described above. We
269 restricted this analysis to combinations of sites and traits where we detected
270 significant linear or quadratic selection.

271 For each site-trait combination, we calculated each genotype's expected
272 change in fitness due to plasticity ($\Delta\omega$) by evaluating the selection differential at
273 two trait values:

274 $$\Delta\omega = f(T_{il}) - f(\bar{T}_l)$$

275 where *f(x)* is the selection differential (i.e., relative fitness as a function of a given
276 trait at a given site; see above), T_{il} is the least-squares mean trait value of
277 genotype *l* at site *i* (depicted by the black and red points in Figure 1g-j), and $\bar{T}_l$ is
278 the least-squares mean trait value of genotype *l* averaged across all sites (depicted
279 by the black and red dashed lines in Figure 1g-j). Both T_{il} and $\bar{T}_l$ were calculated
280 from the REML variance-partitioning model described above. $\bar{T}_l$ represents the
281 trait value of a hypothetical genotype that is identical to genotype *l* except that it
282 lacks plasticity. Therefore, $\Delta\omega$ is an estimate of the fitness change that can be
283 attributed to plasticity among sites of a trait that is under selection. Positive
284 values of $\Delta\omega$ constituted evidence of adaptive plasticity; negative values indicated
285 non-adaptive plasticity.

286 We conducted an exact binomial test of the null hypothesis that plasticity
287 is equally likely to move glucosinolate trait values in an "adaptive" or a "non-
288 adaptive" direction. Weak cases of plasticity, in which the 95% CI of $\Delta\omega$ included
289 zero, were excluded from this analysis.

290 ***Characterizing within-habitat plasticity using reaction norms***

291 In this study, we focused on phenotypic plasticity induced by spatial
292 environmental variation at a single time-point. Because each plant in this study
293 only experienced a single spatial environment, plasticity of individual plants
294 could not be measured. Instead, spatial plasticity of glucosinolate profiles is a
295 property of a genotype, estimated by comparing the phenotypes of individuals
296 that shared the same genotype but were growing in different experimental blocks.
297 To infer whether natural selection was acting on fine-grained glucosinolate
298 plasticity within *B. stricta* habitats, we (1) quantified plasticity among blocks for
299 each genotype as a continuous function or *reaction norm*, and (2) used genotypic
300 selection analysis to test whether reaction norm steepness—a measure of
301 plasticity—predicted evolutionary fitness of each *B. stricta* genotype.
302 First, for each of 25 genotypes we fit one reaction norm to describe each of
303 the three glucosinolate traits as a continuous linear function of an *environmental*
304 *index* (EI, a numerical descriptor of microhabitat conditions within each
305 experimental block)—for a total of 75 reaction norms (3 traits × 25 genotypes).
306 Data from all four sites were pooled for calculation of reaction norms. We
307 assumed that most environmental factors causing glucosinolate plasticity are
308 unknown, and so the relevant environmental characteristics are best "measured"

using plant phenotype data. Therefore, we assigned the grand mean trait values observed in each block (for all 25 genotypes, pooled) to be the environmental indices (Finlay and Wilkinson 1963). We then calculated a reaction norm for each genotype and each trait using linear regression of genotype-specific block mean trait values onto the EIs:

$$Genotype\ trait\ mean\ in\ block = \beta * Block\ environmental\ index + error$$

The linear regression coefficient (β) estimated by each model is a reaction norm *slope*, which describes the magnitude and direction of the plastic response for one genotype. We also calculated the *height* of each reaction norm by evaluating the linear function at the mean EI value; thus, reaction norm height describes the genotype's predicted trait value in an "average" block (Figure 2).

Second, to test whether reaction norm slopes were heterogeneous among genotypes, we analyzed genotype-specific block mean trait values and EIs from all 25 genotypes together by fitting an ANCOVA model with an additional *Genotype* × *EI* interaction term that described genetic variation for reaction norm slopes:

$$Genotype\ trait\ mean\ in\ block = Genotype + Block\ EI + Genotype * Block\ EI + error$$

If the interaction term was significant, we concluded that reaction norm slopes were heterogeneous among genotypes.

Testing for selection on reaction norm slope and reaction norm height

The slope and height of the reaction norms are measurable characteristics of plant genotypes, corresponding to plasticity and average trait values, respectively (Figure 2). We measured linear selection gradients on these reaction norm parameters to determine whether glucosinolate plasticity in response to fine-grained environmental variation affects fitness. Reaction norm parameters were calculated using data pooled from all four sites (see above), but to allow for the possibility that plasticity is not equally advantageous in all habitats, we measured selection on these parameters separately at each site.

To test for linear selection on reaction norm slopes, we conducted genotypic selection analysis separately for each glucosinolate trait at each site. Genotypes' relative fitness within each site (genotypic survival rate × genotypic fecundity, divided by the mean fitness for all genotypes at that site) was regressed onto the genotypes' reaction norm height and slope, generating two partial regression coefficients (β_1 and β_2) that describe linear selection on average trait values and trait plasticity, respectively (Figure 2g-h; Supplementary Methods):

$$Genotype\ relative\ fitness = \beta_1 * RN\ height + \beta_2 * RN\ slope + \beta_3 * Diameter + error$$

The mean rosette diameter for each genotype at time of transplanting, which reflects differences in performance in the greenhouse and not in the field, was included as a nuisance variable.

Table 1: Statistics from REML mixed models of glucosinolate traits reveal strong genotype-by-site interactions. All effects are fixed except for Block, Genotype × Block, and HPLC batch, which were random-intercept terms. Significance of random effects was assessed using likelihood ratio tests. *N*=1,503 for Total [GS]. For BC-ratio and BC-diversity, *N*=1,486 because these traits could not be measured for individuals that produced no glucosinolates.

	BC-ratio	Total [GS]	BC-diversity
R^2	0.99	0.49	0.92
Genotype	$F_{23,1035}$=343.68 ***P*<3e^{-16}**	$F_{23,949}$=9.24 ***P* <3e^{-16}**	$F_{23,982}$=140.98 ***P* <3e^{-16}**
Site	$F_{3,109}$=21.28 ***P* =1.3e^{-10}**	$F_{3,99}$=14.07 ***P* =1.0e^{-07}**	$F_{3,1085}$=49.28 ***P* <3e^{-16}**
Genotype × Site	$F_{69,1035}$=3.93 ***P* <3e^{-16}**	$F_{69,899}$=3.02 ***P* =7.2e^{-14}**	$F_{69,972}$=12.91 ***P* <3e^{-16}**
Dev. Stage	$F_{2,785}$=4.31 ***P* =0.014**	$F_{2,1332}$=23.78 ***P* =2.1e^{-10}**	$F_{2,1226}$=6.49 ***P* =0.0031**
Height	$F_{1,1109}$=33.05 ***P* =2.3e^{-08}**	$F_{1,1344}$=46.86 ***P* =3.5e^{-11}**	$F_{1,1328}$=6.60 ***P* =0.010**
Block	χ^2_1=22.74 ***P* =3.7e^{-06}**	χ^2_1=49.23 ***P* =4.2e^{-14}**	χ^2_1=4.3e^{-11} *P*=1
Genotype × Block	χ^2_1=58.90 ***P* =5.0e^{-14}**	χ^2_1=0.03 *P*=0.86	χ^2_1=12.37 ***P* =0.00087**
HPLC batch	χ^2_1=4.09 ***P* =0.043**	χ^2_1=9.66 ***P* =0.006**	χ^2_1=8.33 ***P* =0.0078**

Results

Genotype and environment synergistically controlled glucosinolate profiles

All three glucosinolate profile features—BC-ratio, Total [GS], and BC-diversity—varied among genotypes and among field sites (Figure 5a; Table 1). All three traits were also affected by developmental stage and plant size (Table 1; Supplementary Figure 4). Genotypic variation for BC-ratio has been previously

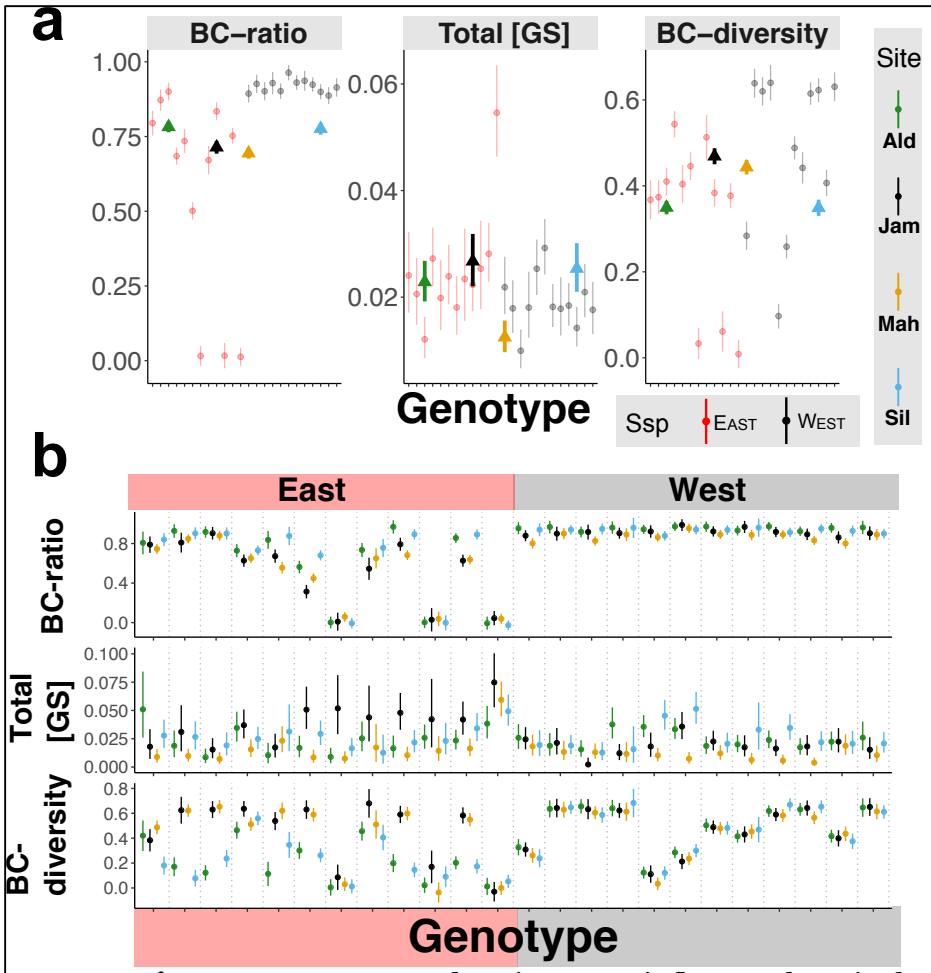

Figure 5: Genotype and environment influence glucosinolate profiles both independently and synergistically. **(a)** Both genotype and habitat influence glucosinolate profiles. Plotted are least-squares mean trait values for each Genotype (circles) and for each Site (triangles) from a REML mixed model that also controlled for developmental stage, plant size, genotype-by-site interactions, and block and batch effects (Table 1). Thus, circles show the mean trait value for each genotype (averaged across all sites); triangles show the mean trait value at each site (average for all genotypes). Note that the horizontal position of the triangles is meaningless—they were placed in order to not obscure the genotype means. Error bars are 95% confidence intervals. **(b)** Glucosinolate plasticity among sites is genetically variable. Genotypes are delimited by vertical dashed lines. The points are least-squares mean trait values for each genotype in each common garden, with 95% confidence intervals. BC-ratio and BC-diversity are unitless; units for Total [GS] are μmol/mg dry tissue.

reported in *B. stricta* (Schranz *et al.* 2009; Manzaneda *et al.* 2010); our data show that Total [GS] and BC-diversity are also genetically controlled. In this experiment the East subspecies harbored more genetic variation for BC-ratio

366 than the WEST; however, genetic variability for the other traits was comparable
367 between the subspecies (Figure 5a). Genotypes varied less in glucosinolate
368 quantity than in BC-ratio and BC-diversity, except for SAD12, which produced
369 nearly twice the concentration of glucosinolates as the others. SAD12 is also
370 notable as the only genotype in the experiment that originated in Colorado; the
371 others are from Idaho or Montana. Nevertheless, analysis of Total [GS] yielded
372 the same results regardless of whether the outlier SAD12 was included.

373 All three traits showed significant plasticity among sites. For Total [GS],
374 the magnitude of the site effect was comparable to variation attributed to
375 genotype—in particular, plants growing at Mahogany Valley produced only 50%
376 the quantity of glucosinolates as those growing at the other sites, on average
377 (Figure 5a). In contrast, for BC-diversity and especially BC-ratio, the magnitude
378 of plasticity among sites was minor compared to the variation due to genotype.
379 Additionally, BC-ratio and Total [GS] varied significantly among blocks within
380 sites (6.3% and 11.2% of the total variance, respectively), indicating that meter-
381 scale environmental heterogeneity affected expression of these traits
382 (Supplementary Figure 5a; Table 1).

383 Finally, significant genotype-by-site interactions confirm that genotype
384 and environment acted synergistically to shape glucosinolate profiles (Table 1). In
385 general, EASTERN genotypes were more sensitive to environment than WESTERN
386 genotypes, especially for BC-ratio and BC-diversity. However, patterns of
387 plasticity among sites varied even within subspecies (Figure 5b). In addition to
388 these genotype × site interactions, genotype × block interactions accounted for
389 65.3% and 35.9% of the variance in BC-ratio and BC-diversity, respectively. This

390 indicates strong genetic variation for plasticity of glucosinolate composition in
391 response to environmental heterogeneity on the meter scale within habitats.
392 Consistent with the observed subspecies difference in plasticity *among* habitats
393 (Figure 5b), EASTERN genotypes displayed greater plasticity among blocks *within*
394 habitats, as well (Supplementary Figure 5b).

395

396 ## *Evidence for frequent adaptive plasticity among sites*

397 ### Selection on glucosinolate profiles in all habitats

398 Phenotypic selection analysis revealed seven cases of linear selection on
399 glucosinolate traits, and two cases of disruptive (quadratic) selection (Figure 6;
400 Supplementary Table 5). High Total [GS] was associated with decreased
401 fecundity in all four common gardens (Figure 6b-e; Supplementary Table 5).
402 Pooling the data from all sites and testing for a Site*Total [GS] interaction failed
403 to reject the null hypothesis that these selection differentials were equivalent
404 (Supplementary Table 6), suggesting that high glucosinolate concentrations are
405 equally costly or disadvantageous in all four habitats. In contrast, linear selection
406 on BC-diversity varied among sites (Supplementary Table 6). Specifically, high
407 BC-diversity was associated with higher fecundity at Jackass Meadow but lower
408 fecundity at Alder Creek and Silver Creek (Figure 6f-h); however, we detected no
409 significant selection on BC-diversity at Mahogany Valley (Supplementary Table
410 5).

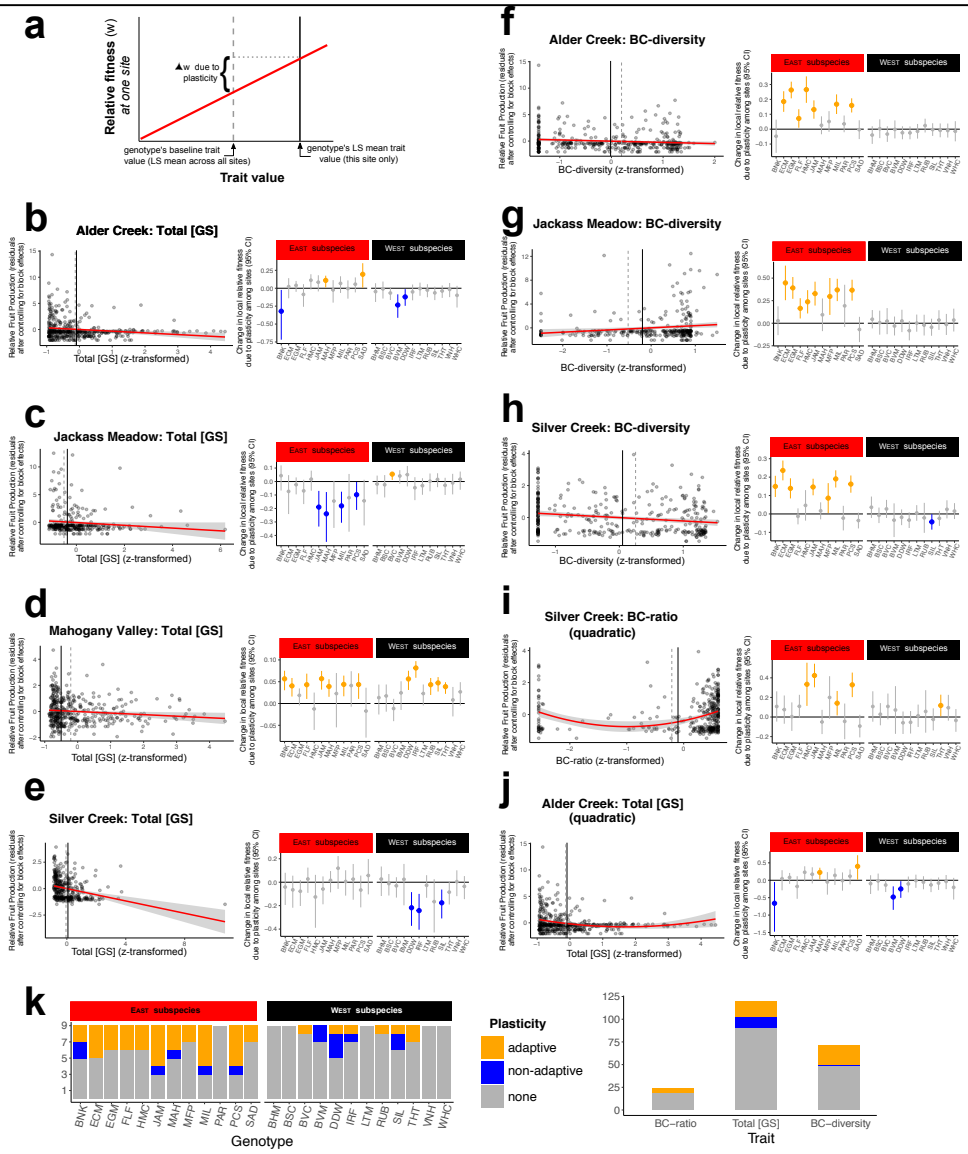

Figure 6: Evidence for adaptive glucosinolate plasticity among field sites. We detected seven cases in which a glucosinolate trait was under linear selection within a field site (**b-h**), and two cases of quadratic (disruptive) selection (**i-j**). Selection differentials are plotted as red lines. For each of these cases, we asked whether patterns of plasticity among sites resulted in increased or decreased relative fitness ($\Delta\omega$). To do this for each genotype, we evaluated the selection differential at two values: the genotype's LS mean trait value across all sites (which represents a hypothetical "no plasticity" scenario) and the genotype's true LS mean trait value at that site **(a)**. The averages of these trait values across all genotypes are depicted as dotted and solid vertical lines, respectively, in the left side of panels (**b-h**). In the right side of panels (**b-h**), $\Delta\omega$ due to plasticity is plotted for each genotype. Positive and negative estimates of $\Delta\omega$ are considered evidence for *adaptive* and *non-adaptive* plasticity, respectively, if their 95% CIs do not contain 0 (colored in orange and blue respectively). Panel (**k**) summarizes counts of adaptive and non-adaptive plastic responses for each genotype (left) and each glucosinolate trait (right). Overall, plasticity was in an adaptive direction more often than would be expected by chance (44 out of 58 observations; exact binomial test, P=0.0001).

411

412 For thoroughness, we also calculated linear selection *gradients* to assess direct
413 selection on each trait while controlling for indirect selection on the other
414 glucosinolate traits (Supplementary Methods). The selection gradients generally
415 agreed with the selection differentials, and also indicated that BC-ratio may be
416 under negative selection at Mahogany Valley (Supplementary Table 7).
417 Finally, we found evidence that disruptive selection is acting on BC-ratio at
418 Silver Creek and on Total [GS] at Alder Creek (Figure 6i-j; Supplementary Table
419 8).

420 Plasticity among habitats was more likely to move glucosinolate 421 profiles in an adaptive than a non-adaptive direction

422 Each of the nine cases in which a glucosinolate trait was under selection at
423 a given site (Figure 6b-j) was an opportunity for plasticity to alter trait values in a
424 way that affected evolutionary fitness. Because the strength and direction of
425 plasticity varied among genotypes (Figure 5), we assessed the frequency of
426 adaptive versus non-adaptive plasticity for each genotype. We used each
427 genotype's experiment-wide LS mean trait values as baselines, representing a
428 hypothetical genotype that was identical except that it lacked plasticity among
429 sites (Figure 1g-k). Relative to this baseline, plasticity was strong enough to
430 substantially alter relative fitness in 27% of all cases. This was especially true for
431 genotypes of the EAST subspecies, which were more plastic than WESTERN
432 genotypes, particularly for BC-ratio and BC-diversity (Figure 5b). Plasticity
433 among sites affected fitness nearly 40% of the time for EASTERN genotypes, but
434 only 14% of the time for WESTERN genotypes (Figure 6k).

435 Of the 58 cases in which a genotype's plasticity was strong enough to affect
436 its fitness, 44 shifted trait values in an adaptive direction—considerably more
437 than would be expected by chance (exact binomial test, P=0.00015; Figure 6k).
438 We found that the direction of plasticity matches the direction of selection 75.9%
439 of the time (95% CI = 62.8% to 86.1%). However, this was largely driven by the
440 EAST subspecies—not only because WESTERN genotypes were less plastic overall
441 (χ^2=13.5, P=0.0007), but also because their plastic responses were more likely
442 than those of EASTERN genotypes to be in a non-adaptive direction (χ^2=9.4,
443 P=0.039; Figure 6k).

444 *Glucosinolate reaction norms were genetically variable but not*
445 *under detectable selection*

446 Reaction norm slopes of all three glucosinolate traits varied strongly
447 among genotypes, indicating substantial genetic variation for glucosinolate
448 plasticity in response to continuous environmental gradients (Figure 7;
449 Supplementary Table 9a). After correction for multiple testing, genotypic
450 selection analysis failed to detect significant selection differentials on reaction
451 norm slopes at any site (Supplementary Table 10), suggesting that glucosinolate
452 plasticity in response to continuous environmental gradients was neither
453 beneficial nor costly within any of these four habitats.

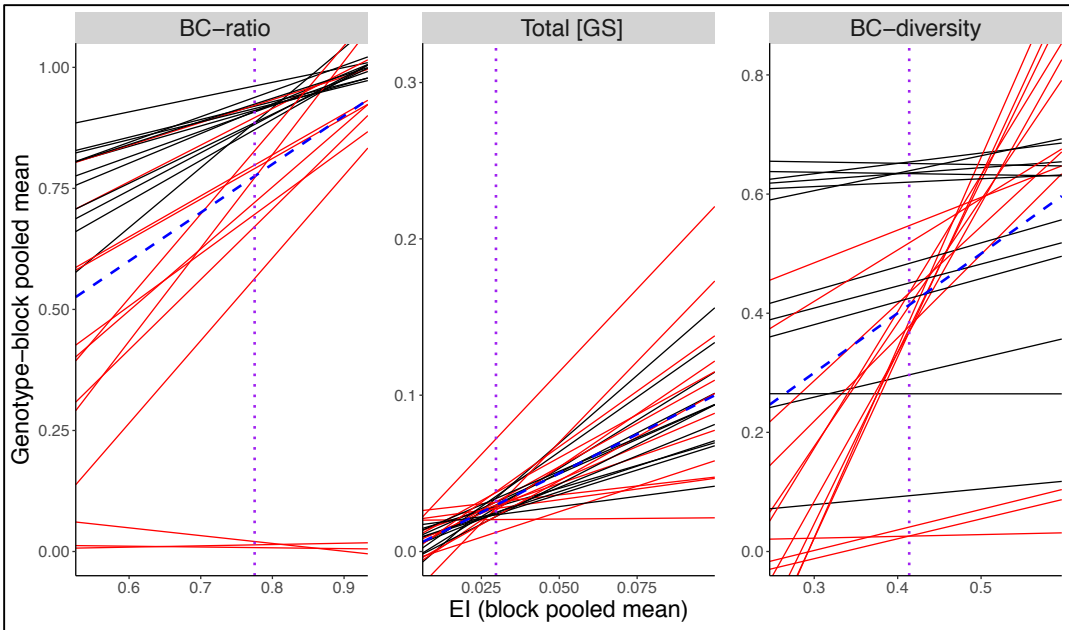

Figure 7: Genotype-specific glucosinolate reaction norms. The mean trait values of each genotype in each block were regressed onto the grand mean trait values for all genotypes in each block, or "environmental index". Each resulting regression line is a reaction norm for one genotype, plotted here in red or black for EASTERN or WESTERN genotypes, respectively. Genotypes with steeper reaction norm slopes exhibit more plasticity in response to continuous environmental gradients. The "average reaction norm" (equivalent to the line $y=1*x$, where the expressed trait value equals the block mean trait value) is shown as a blue dashed line. The purple vertical dotted line denotes the mean environmental index for each trait (i.e., an average environment), the value at which reaction norm height was evaluated.

Discussion

In *Boechera stricta*, plant genotype and environment synergistically affect the concentration and composition of glucosinolate profiles. All three emergent traits of glucosinolate profiles—Total [GS], BC-ratio, and BC-diversity—were under both genetic and environmental control, with strong genotype-by-environment interactions. Genotype was the strongest determinant of BC-ratio and BC-diversity, whereas genotype and environment had similar effect sizes for Total [GS] (Figure 5a). We observed abundant genetic diversity for plasticity both

463 among and within field sites (Figure 5b; Figure 7; Table 1). Particularly striking
464 was the 65% of BC-ratio variation that was explained by genotype-by-block
465 interactions (Supplementary Figure 5b; Table 1).

466 In general, EASTERN genotypes were more plastic than WESTERN
467 genotypes, and there was additional genetic variation in reaction norm shape and
468 inter-site plasticity within the EAST subspecies (Figure 5b; Figure 7). However,
469 this divergence does not reflect adaptation to within-habitat heterogeneity. With
470 a few exceptions, all of the environmental variables that we measured had similar
471 variances in the two EASTERN habitats and the two WESTERN habitats
472 (Supplementary Figure 6), indicating that neither subspecies generally occupies
473 more complex habitats than the other. The lack of variation for glucosinolate
474 plasticity among WESTERN genotypes is consistent with observed patterns of
475 reduced molecular diversity relative to EASTERN genotypes (Baosheng Wang,
476 personal communication), and might limit further evolution of reaction norms
477 within the WEST but not the EAST subspecies.

478 ***Glucosinolate plasticity may aid colonization of new habitats***

479 Because the distances separating our field sites are much greater than the
480 dispersal distance of *Boechera* (Bloom *et al.* 2002), it is unlikely that the inter-
481 site plasticity observed in this study evolved as an adaptation to heterogeneity
482 among sites (Via and Lande 1985; Gomulkiewicz and Kirkpatrick 1992). Instead,
483 the differences between pairs of sites are reasonable simulations of transitions to
484 novel environments, or when streams carry seeds to lower-elevation sites. Thus,
485 we could assess whether this coarse-grained glucosinolate plasticity might impact

486 the likelihood that a *B. stricta* population could survive a major environmental
487 change or colonize a new habitat (Figure 1; Ghalambor *et al.* 2007).
488 Whether plasticity of a trait improves the relative fitness of a population
489 upon encountering a new environment depends on the directions and
490 magnitudes of both natural selection and the plastic response. Characterizing
491 plastic responses in this way requires the definition of a "baseline" against which
492 the site-specific trait values can be compared; here we used each genotype's
493 experiment-wide mean trait value as the baseline. We note that if the four field
494 sites used in this experiment were somehow unrepresentative of the wider range
495 of habitats occupied by wild *B. stricta* populations, then this baseline might not
496 reflect the "true" average glucosinolate profile, and thus our estimations of
497 plasticity might be incorrect. We have no reason to suspect this is the case,
498 because we chose these sites to reflect the diversity of habitat types in the region
499 (Supplementary Figure 1; Supplementary Table 2): Alder Creek and Silver Creek
500 are riparian meadows—representative habitats of the WESTERN subspecies—while
501 the other two sites are typical EASTERN subspecies habitats—dry, high-elevation
502 meadows (Lee and Mitchell-Olds 2011, 2013). Nevertheless, repetition of this
503 experiment in a wider range of *B. stricta* habitats would be necessary to rule out
504 this possibility.
505 In this experiment, we detected natural selection acting on at least one
506 glucosinolate trait at all four field sites (Figure 6b-j). Selection consistently
507 favored lower Total [GS], suggesting a cost of producing these defensive
508 compounds (Mauricio 1998). In contrast, the direction of selection on BC-
509 diversity varied among sites. Strikingly, plasticity of BC-diversity in EASTERN

510 genotypes mirrored these varying selection pressures. Trait values increased in
511 sites where BC-diversity was under positive selection, and decreased where it was
512 under negative selection, resulting in significant fitness boosts for the most
513 plastic genotypes (Figure 6f-h). Across all traits, sites, and genotypes, plasticity
514 was often not strong enough to substantially affect fitness. When it was, however,
515 it was much more likely to increase fitness than to decrease it (Figure 6k; exact
516 binomial test, P=0.00015).

517 The whole of the data suggest that glucosinolate plasticity often changes
518 defensive chemistry to better match the local selection pressures, and therefore
519 might aid *B. stricta* populations in colonizing new habitats (Ghalambor *et al.*
520 2007). Whether such adaptive plasticity promotes glucosinolate evolution in the
521 long term, however, will be a more difficult question to answer (Ghalambor *et al.*
522 2007; Ghalambor *et al.* 2015; Huang and Agrawal 2016). Identifying the
523 environmental cues perceived by the plants that induce changes in defensive
524 chemistry, as well as the ecological causes of local selection pressures, should be a
525 priority for future research on adaptive glucosinolate plasticity.

526 Finally, we highlight the implications of genetic variation for plasticity.
527 Although glucosinolate plasticity was adaptive on average, some genotypes were
528 more likely than others to exhibit nonadaptive plasticity or to simply lack plastic
529 responses (Figure 6k). Based on our results, we expect glucosinolate plasticity to
530 aid the colonization of new habitats 20.4% of the time, overall. However,
531 considering only WESTERN genotypes, this estimate drops to 6.5%, while EASTERN
532 genotypes are expected to benefit from glucosinolate plasticity 34.3% of the time.
533 Similarly, glucosinolate plasticity is expected to *hinder* colonization of new

534 habitats 6.5% of the time species-wide, but the rate is slightly higher for WESTERN
535 than EASTERN genotypes (Figure 6k). These results illustrate that the contribution
536 of glucosinolate plasticity to persistence after environmental change is not
537 uniform across the species.

538 ***No evidence for selection on plasticity within habitats***

539 In this experiment, we detected little evidence for selection on
540 glucosinolate reaction norms, or plasticity in response to continuous
541 environmental gradients. Because this study included only 25 genotypes,
542 evidence for selection on reaction norms may become clearer as more genotypes
543 are analyzed. Consistent with this, selection gradients on reaction norm height
544 (i.e., mean trait values across all blocks) lacked statistical support but agreed
545 qualitatively with the patterns detected using the phenotypic selection analysis
546 (compare β values in Supplementary Table 5 with β_H values in Supplementary
547 Table 10). Another possible reason for this negative result is that selection
548 pressures on glucosinolate profiles may not vary on such a fine spatial scale,
549 reducing the opportunity for adaptive plasticity within habitats (Via and Lande
550 1985; Gomulkiewicz and Kirkpatrick 1992). The lack of observed selection
551 *against* plasticity suggests that glucosinolate plasticity does not carry a
552 significant cost (Auld *et al.* 2010).

553 In addition, inter-annual variation is one potential cause of plasticity and
554 variable selection that we did not address in this study. Other experiments have
555 shown that herbivory pressure on *B. stricta* varies considerably over a span of a
556 few years within a single site (Mitchell-Olds, unpublished); consecutive

557 generations of a *B. stricta* lineage might therefore experience very different
558 predatory environments, potentially causing temporally heterogeneous selection
559 on glucosinolate profiles. The consistent negative selection on glucosinolate
560 quantity in this study (Figure 6b; Supplementary Table 5) suggests that herbivory
561 intensity may have been lower than usual, reducing the usefulness of these
562 expensive phytochemicals (Mauricio and Rausher 1997; Mauricio 1998).
563 Temporal fluctuations in resource availability (e.g., due to limited or abundant
564 rainfall) or pathogen pressure could also affect the relative costs and benefits of
565 glucosinolate profile properties. Because *B. stricta* is a perennial, even a single
566 individual may need to adjust to several environments over its lifetime; in theory,
567 plasticity could evolve as an adaptation to such fine-scale variability
568 (Gomulkiewicz and Kirkpatrick 1992; Reed *et al.* 2010; Baythavong 2011). The
569 relative importance of temporal environmental variation compared to spatial
570 variation could be assessed by extending a similar experimental design over
571 multiple growing seasons. Follow-up studies should prioritize temporal variation
572 as a potential driver of adaptive plasticity in glucosinolate profiles and other
573 important *B. stricta* traits.
574 Importantly, evidence of natural selection on reaction norms would not be
575 sufficient to demonstrate that plasticity evolved as an adaptation. Reliable
576 environmental cues for the different selection pressures are also required,
577 preventing the evolution of plasticity as an adaptation to truly stochastic
578 environmental fluctuations (Levins 1963; Donohue *et al.* 2000; Schmitt *et al.*
579 2003; Reed *et al.* 2010). In other words, if an organism's offspring reliably
580 encounter multiple contrasting selection pressures, and if some perceptible

581 environmental factor predicts how selection will act, plasticity (rather than
582 habitat specialization) will evolve as a long-term adaptation to a heterogeneous
583 environment (Donohue *et al.* 2000; Sultan and Spencer 2002). Even when these
584 conditions are not met, however, plasticity can still improve short-term relative
585 fitness—that is, it may sometimes be "adaptive" even if it did not evolve as an
586 "adaptation" (Sultan 1987).

587 ***Future directions***

588 The molecular basis of genotype-by-environment interactions is a key
589 research goal for understanding the evolution of phenotypic plasticity and the
590 robustness of genetic improvements in crop species (El-Soda *et al.* 2014). The
591 ample genetic variation for plasticity of three glucosinolate traits provides an
592 opportunity to explore this phenomenon at the molecular level in *B. stricta*,
593 which offers resources such as near-isogenic lines varying in glucosinolate
594 synthesis genes, a recently developed genome-wide association population, a fully
595 sequenced genome, and genetic similarity to the model plant *Arabidopsis*
596 *thaliana* (Rushworth *et al.* 2011; Prasad *et al.* 2012; Lee *et al.* 2017). The
597 *Arabidopsis* glucosinolate biosynthetic pathway is well characterized (Halkier
598 and Gershenzon 2006), although genes affecting plasticity are not necessarily
599 part of this core pathway. Indeed, in *Arabidopsis*, patterns of flux through this
600 pathway were robust to several biotic and abiotic environmental stimuli (Olson-
601 Manning *et al.* 2015), suggesting that variation in environment-sensing genes or
602 other upstream genes may be more important for glucosinolate plasticity, *per se*.

603 The exact ecological causes of glucosinolate plasticity in this experiment
604 are still unclear and cannot be determined from this dataset; additional
605 experiments with environmental manipulations will be necessary to identify the
606 causal stimuli (Anderson *et al*. 2014). Many environmental features distinguish
607 the field sites used in this study (Supplementary Figure 1). For this reason, we
608 used plant phenotype to define the environmental index of each block, rather
609 than guessing which environmental parameters were most relevant (Finlay and
610 Wilkinson 1963). The disadvantage of this method is that the phenotype is a
611 "black box" obscuring the actual ecological features driving trait plasticity. The
612 question of which ecological factors drive plasticity of glucosinolate profiles—and
613 selection on them—will be an important step towards understanding the
614 apparent link between natural selection and phenotypic plasticity in diverse
615 habitats across a natural landscape.
616 Finally, it is an open question whether the patterns of adaptive plasticity
617 observed in this experiment are typical of other functional traits. Although
618 glucosinolates are known to be ecologically and evolutionary important, they
619 represent only a fraction of the traits contributing to evolutionary fitness in *B*.
620 *stricta* and other Brassicaceae. Additional experiments are needed to clarify
621 whether the evolutionary implications of glucosinolate plasticity are generalizable
622 to other adaptively important traits in *B*. *stricta* and other study species. Until
623 more examples from wild populations are available, it will remain challenging to
624 identify patterns and draw general conclusions about the role of plasticity in
625 adaptive evolution.

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