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# Macroevolutionary patterns of glucosinolate defense and tests of defense-escalation and resource availability hypotheses

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## Summary

- We explored macroevolutionary patterns of plant chemical defense in *Streptanthus* (Brassicaceae), tested for evolutionary escalation of defense, as predicted by Ehrlich and Raven's plant–herbivore coevolutionary arms-race hypothesis, and tested whether species inhabiting low-resource or harsh environments invest more in defense, as predicted by the resource availability hypothesis (RAH).
- We conducted phylogenetically explicit analyses using glucosinolate profiles, soil nutrient analyses, and microhabitat bareness estimates across 30 species of *Streptanthus* inhabiting varied environments and soils.
- We found weak to moderate phylogenetic signal in glucosinolate classes and no signal in total glucosinolate production; a trend toward evolutionary de-escalation in the numbers and diversity of glucosinolates, accompanied by an evolutionary increase in the proportion of aliphatic glucosinolates; some support for the RAH relative to soil macronutrients, but not relative to serpentine soil use; and that the number of glucosinolates increases with microhabitat bareness, which is associated with increased herbivory and drought.
- Weak phylogenetic signal in chemical defense has been observed in other plant systems. A more holistic approach incorporating other forms of defense might be necessary to confidently reject escalation of defense. That defense increases with microhabitat bareness supports the hypothesis that habitat bareness is an underappreciated selective force on plants in harsh environments.

## Introduction

Integrating phylogenetic hypotheses into the study of plant defense has created opportunities to explore the role of plant defenses in adaptive radiation (Becerra, 1997; Armbruster *et al.*, 2009), coevolution and arms races with specialist herbivores (Agrawal & Fishbein, 2008; Becerra *et al.*, 2009), habitat specialization (Fine *et al.*, 2006), ontogenetic changes (Karinho-Betancourt *et al.*, 2015), and the evolution of community structure (Strauss & Agrawal, 1999; Agrawal, 2007; Becerra, 2007; Futuyma & Agrawal, 2009; Kursar *et al.*, 2009; Pearse & Hipp, 2009). To date, clade-wide studies on the evolution of plant secondary compounds with an explicit phylogenetic framework include terpenes in *Bursera* (Becerra, 1997), nonprotein amino acids and alkaloids in legumes and other families (Wink, 2003, 2013; Wink & Mohamed, 2003; Karinho-Betancourt *et al.*, 2015), flavonoids in *Oenothera* (Johnson *et al.*, 2014), tannins in *Quercus* (Pearse & Hipp, 2009), and cardenolides and phenolics in *Asclepias* (Agrawal *et al.*, 2009; Rasmann & Agrawal, 2011). By contrast, despite their agricultural importance and use as model organisms, clade-wide

studies of chemical defense in *Brassicaceae* have been few (but see Agerbirk *et al.*, 2008). Here, we explore macroevolutionary patterns of glucosinolate chemical defenses in the brassicaceous clade *Streptanthus* (s.l., Thelypodieae; Cacho *et al.*, 2014). We test whether patterns of defense conform to hypotheses on escalation of defense through evolutionary time, as predicted under 'escape-and-radiate' dynamics (Ehrlich & Raven, 1964), and to predictions of the resource availability hypothesis (RAH; Coley *et al.*, 1985) in the context of nutrient analyses of soils from species' sites.

Under 'escape-and-radiate' dynamics (Ehrlich & Raven, 1964), plant defenses and herbivore counter-defenses coevolve in a series of sequential cycles that result in an increase in chemical complexity over evolutionary time, and have been proposed to explain the great diversity of plant secondary chemistry (Ehrlich & Raven, 1964; Berenbaum, 1983). Evidence for evolutionary escalation in plant secondary chemistry is mixed. An analysis of terpenes in *Bursera* showed escalation in the number of defense compounds and in chemical complexity over evolutionary time (Becerra *et al.*, 2009). By contrast, in *Asclepias*, cardenolides decrease, while phenolics and regrowth ability escalate over

evolutionary time (Agrawal & Fishbein, 2008; Agrawal *et al.*, 2009). In Apiaceae, a sequence of increased chemical complexity from hydroxycoumarins to linear furanocoumarins to angular furanocoumarins supports the hypothesis of chemical structural escalation (Berenbaum & Feeny, 1981; Berenbaum, 1983). Under escape-and-radiate dynamics, we predict that the diversity or complexity of glucosinolate compounds in *Streptanthus* should increase over evolutionary time.

Another context in which investment in defense has been explored has to do with the types of habitats in which plants grow. The RAH predicts that plants living in environments that impose high costs of tissue replacement are expected to invest more in defense (Janzen, 1974; Coley *et al.*, 1985; Fine *et al.*, 2006). In a study comparing plant traits across soil and moisture gradients, plants growing in low-nutrient environments tended to have a thicker epidermis and greater production of phenols and tannins relative to nitrogen content (Cunningham *et al.*, 1999). Janzen (1974) was probably the first to suggest that herbivores and plant defenses rather than physiological tolerance to poor soils could drive edaphic specialization. Fine *et al.* (2006) showed that species growing in sandy, nutrient-poor soils invested more in defense than congeners from richer clay soils, and that herbivory and investment in defense may have mediated plant edaphic specialization. In this case, herbivory had greater effects in nutrient-poor white sand habitats, and less well-defended congeners from richer clay soils experienced higher herbivory and low fitness in white sand soils.

In addition to nutrients, the bareness of the environment in which plants grow could be an important contributor to investment in plant defense. There is a growing body of work showing that plants occurring in bare environments are subject to multiple nonexclusive sources of selection (Janzen, 1974; Batten *et al.*, 1979; Wesser & Armbruster, 1991; Armbruster, 2014; Cacho & Strauss, 2014), including drought, disturbance, and herbivory. Increased microhabitat bareness may select for increased allocation to plant defense owing to low water availability, which may make replacing leaf tissue lost to herbivores difficult, as well as to increased plant apparency and enemy pressure through a loss of associational resistance (Feeny, 1976; Burns, 2010; Strauss & Cacho, 2013). In our system, under RAH, we predict an increase in investment in glucosinolates in serpentine soils, which are lower in P and K but equivalent in nitrates (Cacho & Strauss, 2014), and possibly also in barer microhabitats, where water deficit might preclude easy replacement of leaf tissue lost to herbivores.

Here, we relate clade-wide glucosinolate profiles and ecological data on habitat attributes for species in the *Streptanthus* clade (Brassicaceae) to explore patterns in the evolution of glucosinolate defense with an explicit phylogenetic framework. Specifically, we address: patterns of evolutionary conservatism in glucosinolate production; evidence for escalation of glucosinolate defense over evolutionary time; and whether glucosinolate defense is greater in harsh bare environments, in nutrient-poor soils, or in serpentine soil endemics, which comprise about a third of the species in the *Streptanthus* clade.

## Materials and Methods

### The *Streptanthus* clade

The *Streptanthus* clade is a group of *c.* 35–50 species of mustards with their center of diversity in California. Many species are habitat and edaphic specialists (Baldwin *et al.*, 2012) typically occupying bare rocky environments. Up to a third of the species in *Streptanthus* are specialized to serpentine soils (Kruckeberg, 1954) which tend to be low in Ca : Mg ratios and nutrient (P, K) concentrations, and have high amounts of toxic elements (Ni, Cr, and Co; Brady *et al.*, 2005). Serpentine use has evolved between four and five times in *Streptanthus* (Cacho *et al.*, 2014), and serpentine sites used by streptanthoids are significantly less vegetated than nonserpentine ones (Cacho & Strauss, 2014). We have recently shown that ancestral occupation of bare habitats may have served as an evolutionary precursor to serpentine soil use in this group (Cacho & Strauss, 2014). Thus, one axis in which *Streptanthus* experience environments where tissue replacement may be costly is the occupation of serpentine soils, while a second axis of harshness may be inhabiting rocky, unvegetated habitats in general, where plants may experience increased drought (Brady *et al.*, 2005), higher rates of disturbance and erosion (Walker, 1954; Rogers & Schumm, 1991; Kruckeberg, 2002; Brady *et al.*, 2005), and increased exposure to UV. Additionally, *Streptanthus* generally suffer high rates of herbivory from both specialists and generalists (Shapiro, 1981a; Courtney, 1986), and we have shown that streptanthoids inhabiting naturally or experimentally barer microsites receive more damage than plants with neighbors (Strauss & Cacho, 2013). The variation in nutrients and bareness across the sites occupied by species in this clade, the availability of a well-resolved phylogeny (Cacho *et al.*, 2014), and the presence of a diversity of antiherbivore defenses – glucosinolates (Rodman *et al.*, 1981; Davis & Boyd, 2000), pierid egg-mimicry (Shapiro, 1981b), nickel hyperaccumulation (Boyd *et al.*, 1994), and leaf crypsis (Strauss & Cacho, 2013) – make the *Streptanthus* clade an ideal system to test theories about the evolution of plant–herbivore interactions, specifically, evolutionary escalation theory and the RAH.

### Glucosinolates

These amino acid-derived metabolites are synthesized mainly by mustards and close relatives (Rodman *et al.*, 1998; Mithen *et al.*, 2010). Plant injury triggers glucosinolate hydrolysis by myrosinase enzymes, producing toxic compounds whose main role is to mediate plant interactions with herbivores (Kliebenstein *et al.*, 2005; Hopkins *et al.*, 2009), pathogens (Bednarek *et al.*, 2009; Cantor *et al.*, 2011), and competitors (through allelopathy; Lankau & Kliebenstein, 2009; Cantor *et al.*, 2011). The molecular pathways for glucosinolate production and hydrolysis and the genetic basis of their variation have been worked out in *Arabidopsis* (Kliebenstein *et al.*, 2001; Schranz *et al.*, 2007), and studies taking advantage of the genomic resources in *Arabidopsis* and *Brassica* are quickly advancing (Schranz *et al.*, 2006; Mitchell-Olds *et al.*, 2008; Zang *et al.*, 2009; Blair *et al.*, 2012;

Yang *et al.*, 2014). Over 130 different glucosinolates have been identified (Fahey *et al.*, 2001; Kliebenstein *et al.*, 2001; Agerbirk *et al.*, 2008; Hopkins *et al.*, 2009) and can be classified based on whether they are derived from methionine (aliphatic) or a branched-chain amino acid (valine, leucine, isoleucine), the degree of elongation of the amino acid chain (C3 and C4 being common in *Brassica* species analyzed to date), and modifications to the side chain (e.g. alkenylation, hydroxylation, etc.). Other classes of glucosinolates include indolics (tryptophan-based), and aromatics (phenylalanine, tyrosine-based). Branched-chain glucosinolates show inverse expression with aliphatics in *Boechera* and the production of these two classes is determined by single gene polymorphism (Schranz *et al.*, 2007). In *Streptanthus*, we also find this inverse expression in the relative production of branched-chain and aliphatic glucosinolates (see Results).

### Tissue collections

We collected leaf samples in the field from undamaged rosette or cauline leaves of one to five individuals per population for 41 streptanthoid species, with replicate populations per species where possible (one to four populations per species, mean = 1.7). An average of five hole punches of tissue per plant were promptly placed in 400  $\mu$ l of 90% methanol to inactivate the myrosinase enzyme, and five hole punches from the same sample were dried for estimating biomass. When leaves were too small or too divided to take hole punches, we cut them in half, putting one half in methanol and saving the other half for dry mass estimation.

A strength of our field approach is that our samples reflect investment in glucosinolates in undamaged leaves under the abiotic and biotic conditions of the real field environments experienced by species. One disadvantage of field collections is that expression of glucosinolates might be more variable (Booth & Walker, 1992; Wentzell & Kliebenstein, 2008); however, compound identities and profiles are often maintained across environments (Kerwin *et al.*, 2015). Using a subset of populations for which we had leaf samples from both plants growing in natural sites and plants grown in a common glasshouse environment, we found that both total glucosinolate amounts and number of compounds were correlated across these environments, despite a small sample size (total glucosinolate production,  $r=0.51$ ,  $P$ -value = 0.05; number of glucosinolate compounds,  $r=0.47$ ,  $P$ -value = 0.08;  $n=15$ ). Environmental variability should reduce our ability to detect phylogenetic signal, so our estimates in this regard are conservative.

### Glucosinolate extraction, quantification, and identification

Tissue was macerated with two metal ball bearings per tube by shaking samples for *c.* 4 min. Samples were extracted in a 96-well plate format system as in Kliebenstein *et al.* (2001). Analyses of desulphoglucosinolates were carried out on a high-performance liquid chromatograph using a diode array detector (Kliebenstein *et al.*, 2005). Identification of compounds was based on peak retention time and absorbance spectrum, and concentrations

were estimated from areas under the curves using published response factors (Reichelt *et al.*, 2002) as in Supporting Information Table S1, and normalized by DW of the sample.

We calculated total glucosinolate production as the sum of the amounts of all glucosinolate compounds. We also broke down the total glucosinolate amounts by main classes (aliphatic, branched-chain, indolic, aromatic), and calculated the fraction of the total glucosinolates comprising each of these classes. We defined glucosinolate richness for the  $k^{\text{th}}$  species ( $S_k$ ) as the number of unique compounds produced by that species. Also, as a measure of relative investment in individual compounds, we assessed glucosinolate chemical diversity with Shannon–Wiener's  $H$  ( $H = -\sum[(p_i) \times \log_e(p_i)]$ , where  $p_i$  = proportion of total sample represented by compound  $i$  for each species, averaging across populations where multiple populations were sampled. Because evolutionary escalation in defense might also be manifested as increased chemical complexity, we scored a chemical complexity index (CCI) as in Becerra *et al.* (2009). This CCI is calculated as the sum of two Shannon indices, one based on the presence–absence of glucosinolate classes ( $H_{\text{CATEGORY}}$ ), and the other on the relative concentrations of individual glucosinolate compounds ( $H_{\text{RELATIVE}}$ ).

### Soil characterization and microhabitat data

We collected samples from the top 30 cm of soil from randomly selected plants per population ( $n=3$ ) for 116 populations (45 species) and had them chemically analyzed for nitrates ( $\text{NO}_3\text{N}$ ), and Olsen's P and K at the University of California, Davis Analytical Laboratory (<http://www.anlab.ucdavis.edu>). We assigned each soil to one of two discrete categories (serpentine or nonserpentine) based on literature, herbarium records, and field observations of the floristic composition of the area. In a discriminant function analyses of soil chemical profiles, our soil type assignments were over 99% accurate (Cacho & Strauss, 2014).

We estimated microhabitat 'bareness' as the percentage of bare ground or rock in a 25 cm<sup>2</sup> quadrat centered on a *Streptanthus* plant in the field, measuring five to 15 individuals per population, and replicate populations per species when possible. For additional details on protocols, see Cacho & Strauss (2014).

### Comparative methods for glucosinolate evolution

Using species averages of glucosinolate profiles (across population-level averages), and 41 species of Streptanthoids, we tested for phylogenetic conservatism in the total amount of glucosinolates, amounts by structural classes of glucosinolates and their proportions, glucosinolate richness ( $S$ ), diversity (Shannon's  $H$ ) and chemical complexity (CCI). We used Blomberg's  $K$  (Blomberg *et al.*, 2003), a standard measure of phylogenetic signal for continuous characters, which takes a value of zero ( $K=0$ ) when there is a lack of correspondence between a trait's evolution and the phylogeny (no phylogenetic signal) and a value of  $K=1$  when a trait evolves along a phylogeny according to a Brownian motion model of trait evolution. Values of  $K>1$  imply a tighter correlation between phylogeny and the evolution

of a trait than would be expected under a Brownian motion model. We tested significance in  $K$  being different from zero by comparing the empirical  $K$ -values to values derived for 1000 null models that randomly shuffle taxa across the tips of the phylogeny with the function `PHYLOSIGNAL` from the R (R Core Team, 2014) package `PICANTE` v.1.6 (Kembel *et al.*, 2010). To integrate over uncertainty in the phylogenetic inference, we repeated our calculations over 1000 trees randomly selected from the posterior distribution of a 50 million generation Bayesian phylogenetic analysis (Cacho *et al.*, 2014).

### Is there evidence of evolutionary escalation in plant defense?

We first calculated ancestral states for glucosinolate production (total amount), richness ( $S$ ), diversity (Shannon's  $H$ ), and chemical complexity (CCI). Then, using linear models, we explored how these three attributes vary through evolutionary time, measured in branch length units (substitutions per site) derived from the maximum credibility tree from a Bayesian phylogenetic analysis of 50 million generations (Cacho *et al.*, 2014). We calculated maximum likelihood estimates for ancestral states using the function `ACE` (ML method) in the R package `APE` (Paradis *et al.*, 2004); linear models were also implemented in R.

Because microhabitat bareness is correlated with glucosinolate richness in our phylogenetically controlled analyses (see the Results section), is conserved across this clade (Cacho & Strauss, 2014), and increases over evolutionary time (see Results), we incorporated bareness into our analyses of the evolutionary trajectory of glucosinolate richness. Our standardized least-squares model of glucosinolate richness included ancestral states in bareness, evolutionary time, and their interaction as explanatory terms, and was run in `JMP PRO` v.11 (SAS Institute Inc., Cary, NC, USA). An analysis of residuals from the model glucosinolate richness  $\sim$  bareness against time yielded results with the same significant relationship (not shown).

### Glucosinolate evolution and a test of the resource availability hypothesis (RAH)

The RAH predicts greater amounts of glucosinolate defense in environments that impose harsh conditions for plant growth. In *Streptanthus*, several aspects of the abiotic environment might constitute conditions under which replacing plant tissues are costly, among them poor-nutrient soils, dry bare environments, and soils with a constellation of unusual elemental composition, such as ultramafic serpentine (e.g. low Ca:Mg ratio and high nickel). We used replicate sites per species, averaging across population-level averages of soil nutrients ( $\text{NO}_3\text{N}$ , P, K) and glucosinolate defense chemistry. In total we surveyed 45 populations (30 species; Table S2) for all data – glucosinolates, bareness, soil type (serpentine/nonserpentine), and soil nutrient composition ( $\text{NO}_3\text{N}$ , P and K). Two species in our dataset (*Caulanthus amplexicaulis*, *Streptanthus tortuosus*) have populations on and off serpentine, which we kept separate in our RAH analyses owing to

soil and habitat differences. Thus, for our RAH analyses, our dataset consists of 32 samples.

Different classes of glucosinolate compounds may confer different antiherbivore properties (Giamoustaris & Mithen, 1995; Rojas, 1999; Mewis *et al.*, 2005), and thus we analyzed total glucosinolate amounts and amounts by structural glucosinolate class, in addition to glucosinolate richness, diversity, and complexity, in relation to soil  $\text{NO}_3\text{N}$ , P and K. We analyzed  $\text{NO}_3\text{N}$  because this represents the form of nitrogen available to plants.

To assess relative investment in glucosinolates, we used phylogenetic generalized least-squares models (PGLSs; Grafen, 1989), with the `PGLS` function in the R package `CAPER` (Orme *et al.*, 2013). Phylogenetic relationships were derived from the maximum credibility tree from Cacho *et al.* (2014), assuming that variation between tips accumulates in proportion to branch length ( $\lambda$  set to 1). To conform to expectations of normality, bareness values were arcsine-square-root-transformed. To adjust for a one-tailed test ( $\alpha = 0.1$ ) given that we had an *a priori* prediction that investment in glucosinolates should increase in low-nutrient, low-moisture serpentine or bare environments, we divided our  $P$ -values by 2 and kept  $\alpha = 0.05$ . We also adjusted each of our tests for multiple comparisons applying a Bonferroni correction across glucosinolate classes ( $n = 4$ ), aliphatic classes ( $n = 3$ ), fractions ( $n = 4$ ), and diversity metrics ( $n = 3$ ).

### Multivariate approaches investigating the relationships of glucosinolates and environment bareness, serpentine use, and nutrients

We analyzed the partitioning of variance of our glucosinolate data (consisting of the amounts of all individual compounds) with respect to soil type (serpentine binary factor), bareness, and nutrients (continuous variables) using permutational multivariate analysis of variance (9999 permutations) with the function `ADONIS` in the R package `VEGAN` v.2.0-10 (Oksanen *et al.*, 2013). We analyzed models in which our glucosinolate multivariate distance matrix ( $G$ ) was evaluated as a function of:  $S$ ,  $B$ ,  $S*B$ ,  $\text{NO}_3\text{N}$ ,  $P$ ,  $K$ ,  $\text{NO}_3\text{N}*B$ ,  $P*B$ , and  $K*B$ , where  $S$  is a vector of soil type (serpentine–nonserpentine),  $B$  is a vector of bareness, and  $\text{NO}_3\text{N}$ ,  $P$ ,  $K$ , are vectors of each of these soil nutrients. In these analyses terms are added sequentially, so we also explored how the order of terms influenced their relevance in explaining variation in  $G$ .

We visualized glucosinolate profiles using nonmetric multidimensional scaling (NMDS) plots and assessed how soil and bareness relate to glucosinolate profiles by overlaying a serpentine binary factor, and by evaluating the fit of vectors of bareness,  $\text{NO}_3\text{N}$ ,  $P$  and  $K$ , onto our NMDS projections (R package `VEGAN`, as above). We used analysis of similarity (ANOSIM; `ANOSIM` R function) to evaluate whether the binary habitat categorization (serpentine–nonserpentine) could be captured in NMDS space, and searched for a function that would be effective at classifying our glucosinolate profiles as serpentine–nonserpentine using a discriminant function analysis (DFA) in `JMP PRO` v.11 (SAS Institute Inc.).



## Results

We identified a total of 35 glucosinolate compounds in the leaves of streptanthoids (Table S1). Most glucosinolates identified are methionine-derived aliphatics (12 compounds; 34%). Indolic, aromatic, and branched-chain glucosinolates are equally represented (six compounds each, 17%), and unknown sulfated compounds represent *c.* 14% (five compounds). Thirty-two compounds are found in three or more species, and will be the focus of this paper.

### Is glucosinolate defense evolutionarily conserved?

Glucosinolate overall total production is generally evolutionarily labile ( $K=0.54$ , ns; Table 1; Fig. S1) but production per structural class shows significant phylogenetic signal for branched-chain glucosinolates ( $K=1.1$ ,  $P=0.03$ ; Table 1; Fig. S1), C4 aliphatics ( $K=0.94$ ,  $P=0.002$ ), and alkenyl aliphatics ( $K=0.62$ ,  $P=0.048$ ). By contrast, the composition and relative investment in different types of compounds are more conserved (Fig. S2). Relative amounts of aliphatic and branched-chain glucosinolates have significant phylogenetic signal ( $K_{\text{Fraction Aliphatic}}=0.79$ ,  $P=0.007$ ;  $K_{\text{Fraction Branch chain}}=0.89$ ,  $P=0.05$ ; Table 1; Fig. S2).

We found significant phylogenetic signal for glucosinolate richness ( $S$ ) ( $K_S=0.82$ ,  $P=0.04$ ; Table 1), but not for diversity (Shannon's  $H$ ) or chemical complexity (CCI). Individual glucosinolates vary in the phylogenetic signal they exhibit (Table S3).

### Is there evidence for escalation of plant defense over evolutionary time?

Total glucosinolate production is not correlated with evolutionary time ( $P=0.94$ , Table 2) but there is an evolutionary increase in the relative investment in aliphatic glucosinolates

(estimate = 212.5,  $P \leq 0.001$ ; Table 2; Fig. 1) across most of the clade, with a concomitant de-escalation in branched-chain glucosinolates (estimate = -126.8,  $P \leq 0.001$ ; Table 2; Fig. 1). Species of the *Caulanthus* and *Guillenia* subclades do not conform to this pattern because they invest in branched-chain glucosinolates (see Figs S1, S2). Aliphatics are produced in large quantities and by most streptanthoids (Fig. S1), comprising an average of 34% of the total glucosinolates produced across species.

Glucosinolate diversity (Shannon's  $H$ ) and chemical complexity (CCI) show a significant de-escalation trend (Shannon's  $H$  estimate = -181.6,  $P \leq 0.001$ ; CCI estimate = -385.1,  $P \leq 0.001$ ; Table 2, Fig. 1). Glucosinolate richness also decreases with evolutionary time (estimate = -911.4,  $P=0.001$ ; Table 3; Fig. 2) but only when microhabitat bareness is considered in the model.

In sum, we find a general pattern of evolutionary de-escalation in the number and diversity of glucosinolates produced by streptanthoids, and a general increase in the relative dominance of aliphatics.

### Do plants from low-nutrient or otherwise harsh environments invest more in glucosinolate defense? A test of the RAH

We find mixed support for the RAH, which predicts increased investment in defense in environments where tissue replacement is costly. The expectation under the RAH is a negative relationship between glucosinolate production and soil nutrients. The support for the RAH appears to be element- and glucosinolate-dependent. Total glucosinolate production did not covary with soil nutrients examined ( $\text{NO}_3\text{N}$ , P, K; Table 4) but several patterns in support of the RAH arise when analyzing glucosinolate classes (main and aliphatic subclasses), fractions, and diversity

**Table 1** Phylogenetic signal in glucosinolate classes, richness and diversity, in *Streptanthus* s.l.

Variable kind	Variable	<i>K</i>	<i>P</i>	<i>K</i> 95% CI	<i>P</i> 95% CI
Total	Total overall	0.544 ns	0.415	(0.413–0.651)	(0.165–0.832)
Class (main)	Total aliphatic	0.498 ns	0.371	(0.371–0.615)	(0.142–0.807)
	Total aromatic	0.540 ns	0.368	(0.403–0.680)	(0.132–0.652)
	Total branched chain	<b>1.098 **</b>	<b>0.030</b>	<b>(0.924–1.473)</b>	<b>(0.014–0.058)</b>
	Total indolic	0.408 ns	0.814	(0.288–0.536)	(0.536–0.932)
Class (aliphatic)	Total C3	0.550 ns	0.247	(0.427–0.661)	(0.127–0.453)
	Total C4	<b>0.941 **</b>	<b>0.002</b>	<b>(0.765–1.081)</b>	<b>(0.001–0.004)</b>
	Total alkenyl	<b>0.626 **</b>	<b>0.050</b>	<b>(0.490–0.753)</b>	<b>(0.016–0.215)</b>
Fraction	Fraction aliphatic	<b>0.792 **</b>	<b>0.007</b>	<b>(0.649–0.929)</b>	<b>(0.002–0.020)</b>
	Fraction aromatic	0.525 ns	0.371	(0.413–0.636)	(0.209–0.636)
	Fraction branched chain	<b>0.895 **</b>	<b>0.039</b>	<b>(0.723–1.108)</b>	<b>(0.012–0.128)</b>
	Fraction indolic	0.546 ns	0.350	(0.432–0.664)	(0.200–0.566)
Diversity	Richness ( <i>S</i> )	<b>0.819 **</b>	<b>0.041</b>	<b>(0.690–0.927)</b>	<b>(0.016–0.101)</b>
	Richness ( <i>S</i> <sub>25</sub> )	0.611 ns	0.303	(0.493–0.710)	(0.188–0.503)
	Shannon's <i>H</i>	0.849 ns	0.190	(0.739–0.952)	(0.091–0.360)
	Chemical complexity index	0.833 ns	0.208	(0.708–0.941)	(0.095–0.424)

Values of *K* and its *P*-values were derived from 1000 replicates over 1000 post-burn-in randomly sampled trees from Cacho *et al.* (2014), using the function PHYLOSIGNAL from the package PICANTE v.1.6 (Kembel *et al.*, 2010) in R (R Core Team, 2014). Medians and 95% confidence intervals (CIs) are reported. Carbon chain length of aliphatic glucosinolates is indicated by superscripts (C3, C4). Significance at  $\alpha \leq 0.05$  is denoted as \*\* and highlighted in bold; ns, non-significant.

**Table 2** Glucosinolate patterns in *Streptanthus* s.l. through evolutionary time

Variable	All nodes ( $n = 32$ )			
	Intercept	Estimate	adj $R^2$	$P$ -value
Bareness	<b>1.270</b>	<b>130.572</b>	<b>0.141</b>	<b>0.019</b>
Total	3.654	-36.623	-0.033	0.949
Aliphatics total	3.328	287.076	-0.020	0.542
Aliphatics fraction	<b>0.918</b>	<b>212.534</b>	<b>0.287</b>	$\leq 0.001$
Branched chain total	<b>-0.049</b>	<b>-654.762</b>	<b>0.3440</b>	$\leq 0.0003$
Branched chain fraction	<b>0.024</b>	<b>-126.778</b>	<b>0.284</b>	$\leq 0.001$
Alkenyl	2.063	136.943	-0.031	0.779
Shannon $H$	<b>0.792</b>	<b>-181.618</b>	<b>0.500</b>	$< 0.001$
Chemical complexity	<b>1.126</b>	<b>-385.126</b>	<b>0.570</b>	$< 0.001$

Linear models were implemented in R using ancestral states estimated with the function `ACE` (maximum likelihood (ML) method) in the R package `ape` (Paradis *et al.*, 2004), and the maximum credibility tree from a 50 million generation Bayesian analysis (Cacho *et al.*, 2014). Time is in branch length units (substitutions per site). Significant relationships are highlighted in bold.

metrics. Aliphatic C3 and alkenyl glucosinolates are inversely related to all  $\text{NO}_3\text{N}$ , P, and K, but only the relationships C3 with P (estimate = -0.83, adj  $r^2 = 0.1$ ,  $P = 0.02$ ), and alkenyls with K (estimate = -1.62, adj  $r^2 = 0.09$ ,  $P = 0.03$ ) remain significant after correcting for multiple aliphatic subclasses (Table 4). Aromatic compounds and chemical complexity (CCI) are also greater in plants where soils are low in nitrates, and aliphatics increase with decreasing soil P (Table 4). Contrary to RAH predictions, total branched-chain glucosinolates correlate with P and K, as expected from their negative relationship with aliphatics. In addition, the indolic fraction increases with increasing K. Out of 14 significant relationships that we found, 10 are negative, an

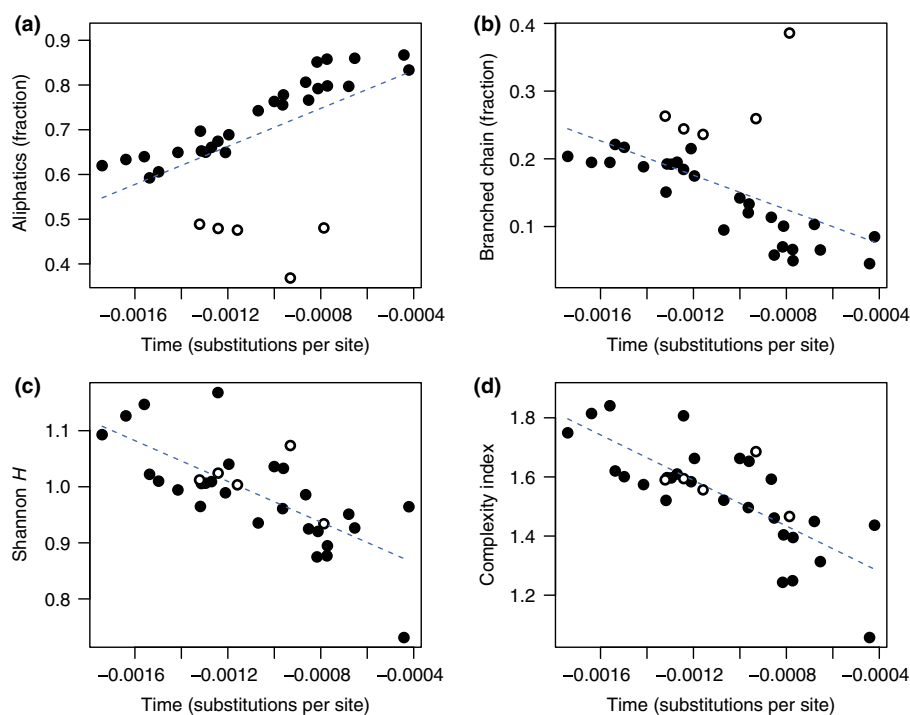
unlikely number to occur by chance (binomial expansion test,  $P = 0.01$ ). Thus, the preponderance of inverse relationships between glucosinolate defense and soil nutrients provides support for RAH.

If bare, more droughty environments represent conditions where tissue replacement is costly, then we also find some support for the RAH. A greater number of glucosinolate compounds are synthesized in barer environments (richness estimate = 4.68, adj  $r^2 = 0.13$ ,  $p_{\text{PGLS}} = 0.011$ ; Table 5; Fig. 2), and this pattern holds when adjusting for four diversity classes tested ( $p_{\text{PGLS}} = 0.045$ ). There is, however, no evidence for more total glucosinolates in bare environments.

Serpentine soils, which are not only bare, but also have high concentrations of metals and low Ca : Mg ratios, might also select for increased defense under the RAH. However, we found no difference in glucosinolate diversity or overall production in serpentine vs nonserpentine soils (soil type estimate = 0.44, adj  $r^2 = -0.02$ ,  $p_{\text{PGLS}} = 0.3$ ; Table 5). That said, plants occupying serpentine environments produce more C4 and alkenyl glucosinolates (Fig. 3; Table 5).

Permutational multivariate ANOVAs indicate field bareness (B), but not serpentine (S), as a significant predictor of glucosinolate profiles (G; Table S4), and this result is robust to the order in which terms are added in the models. In models including soil nutrients, bareness was always significant, even when included as a second term (Table S4). The only soil nutrient examined that explained some variation in our glucosinolate multivariate matrix (G) was K, and only when included as a single or first term if bareness was also in the model (Table S4).

Glucosinolate profiles from populations with similar field microhabitat bareness are closer in NMDS space (the projection of a bareness vector over NMDS is significant;  $R^2 = 0.58$ ,



**Fig. 1** (a, b) Total glucosinolate production in *Streptanthus* s.l. does not vary through evolutionary time but relative investment in aliphatics increases (a), with a concomitant decrease in branched-chain glucosinolates (b). The five points that do not conform to this pattern belong to clades with high branched-chain producing species (*Caulanthus* and *Guillenia* subclades, labeled and highlighted in Supporting Information Fig. S1). (c, d) Glucosinolate diversity (c) and structural complexity (d) also decrease through evolutionary time. Linear models were implemented using ancestral states estimated with the function `ACE` (ML method) in the R package `ape` (Paradis *et al.*, 2004), and the maximum credibility tree from a 50 million generation Bayesian analysis (Cacho *et al.*, 2014). Time is in branch length units (substitutions per site). Dotted lines represent the model fit ( $n = 32$ ). For estimates and  $P$ -values, see Table 2.

**Table 3** Richness de-escalates over evolutionary time in *Streptanthus* s.l. after accounting for an evolutionary increase of microhabitat bareness

Parameter Estimates Term	Estimate	SE	t Ratio	Prob >  t
Intercept	0.530	1.211	0.44	0.665
Bareness	7.268	0.949	7.66	< 0.0001
Time	-911.393	255.249	-3.57	0.001
(Time + 0.00109)* (Bareness - 1.12836)	1088.273	2438.353	0.45	0.659

We analyzed the model glucosinolate richness ~ bareness + time + bareness × time using generalized least squares as implemented in JMP PRO v.11 (SAS Institute Inc.). Ancestral states were estimated with the function ACE (restricted maximum likelihood (REML) method) in the R package APE (Paradis *et al.*, 2004), and the maximum credibility tree from a 50 million generation Bayesian analysis (Cacho *et al.*, 2014). Time is in branch length units (substitutions per site).

$P = 0.006$ ; Fig. S3). K is the only soil nutrient with a significant projection onto NMDS space ( $R^2 = 0.53$ ,  $P = 0.009$ ). An analysis of similarity (ANOSIM) on glucosinolate profiles cannot detect significant differences in glucosinolate profiles between serpentine and nonserpentine species ( $P = 0.43$ ), yet a discriminant

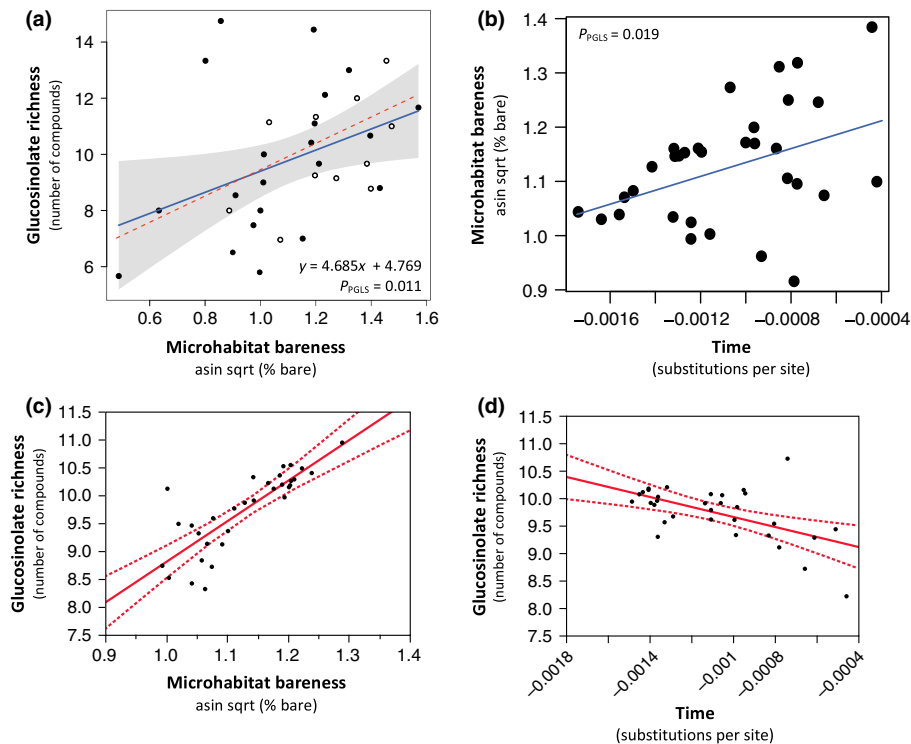
function to categorize population samples according to soil type based on glucosinolate profiles has a 4.5% misclassification (term coefficients in Table S5), suggesting some systematic multivariate differences across soil types.

## Discussion

A strength of our approach is that it integrates phylogenetic history with information on contemporary environments, substrates, and plant defense in the biotic and abiotic contexts where plants occur naturally, across a whole clade.

### Macroevolutionary patterns in glucosinolates and a test of the defense-escalation hypothesis

Total glucosinolate production contained low phylogenetic signal in *Streptanthus*. Lack of conservatism in chemical defense could result from multiple nonexclusive sources of variation, including abiotic factors or inducibility (Kim & Jander, 2007; Himanen *et al.*, 2008), or conflicting selection for different compounds imposed by specialist/generalist herbivores or conspecific/heterospecific competitors (Kliebenstein *et al.*, 2001; Lankau &



**Fig. 2** (a) Glucosinolate richness in *Streptanthus* s.l. is correlated with microhabitat bareness taking phylogeny into account (estimates in Table 5). (b) Microhabitat bareness increases over evolutionary time (see Table 2). (c, d) A model for glucosinolate richness evolution that uses maximum likelihood (ML) ancestral states estimates and incorporates microhabitat bareness, evolutionary time and their interaction as explanatory variables (richness ~ bareness + time + bareness × time) reveals a de-escalation trend in glucosinolate richness through evolutionary time (leverage plots shown; for model estimates, see Table 3). Solid lines are nonphylogenetically corrected models; dotted lines correspond to phylogenetic generalized least-squares (PGLS) model in (a), and to 5% confidence curves in (c) and (d). PGLS models and ancestral states were estimated using the functions PGLS and ACE (ML method) from the R packages CAPER (Orme *et al.*, 2013) and APE (Paradis *et al.*, 2004), respectively, and the maximum credibility tree from a 50 million generation Bayesian analysis (Cacho *et al.*, 2014). Standardized least-squares models were implemented in JMP PRO v.11 (SAS Institute Inc.). Time is in branch length units (substitutions per site). PGLS  $P$ -value was divided by 2 to adjust for a one-tailed test as a result of our *a priori* hypothesis of higher defense in harsher environments. asin sqrt, arcsine-square-root-transformed.



**Table 4** Relationships between glucosinolate chemical defense and soil macronutrients (NO<sub>3</sub>N, P, K) in *Streptanthus* s.l

Variable kind	Variable	NO <sub>3</sub> N				Olsen P				K			
		Estimate	Adj <i>r</i> <sup>2</sup>	<i>P</i>	RAH	Estimate	Adj <i>r</i> <sup>2</sup>	<i>P</i>	RAH	Estimate	Adj <i>r</i> <sup>2</sup>	<i>P</i>	RAH
Total (main classes)	Total	−0.329	−0.014	0.227		−0.591	0.01	0.130		0.037	−0.033	0.484	
	Aliphatic	−0.484	0.015	0.117		<b>−0.912</b>	<b>0.085</b>	<b>0.029</b>	<b>y</b>	−0.800	−0.002	0.171	
	Branched chain	0.175	−0.002	0.171		<b>0.343</b>	<b>0.049</b>	<b>0.058</b>	<b>n</b>	<b>0.609</b>	<b>0.056</b>	<b>0.052</b>	<b>n</b>
	Indolic	−0.021	−0.027	0.341		−0.010	−0.032	0.437		0.225	0.119	0.015	*
	Aromatic	−0.009	−0.032	0.414		<b>−0.070</b>	<b>0.04</b>	<b>0.070</b>	<b>y</b>	−0.098	0.016	0.115	
Aliphatic subclasses	C3	<b>−0.553</b>	<b>0.051</b>	<b>0.057</b>	<b>y</b>	<b>−0.828</b>	<b>0.097</b>	<b>0.023</b>	<b>y*</b>	<b>−1.007</b>	<b>0.033</b>	<b>0.081</b>	<b>y</b>
	C4	−0.146	−0.018	0.250		−0.187	−0.015	0.236		−0.494	0.009	0.132	
	Alkenyl	<b>−0.610</b>	<b>0.039</b>	<b>0.071</b>	<b>y</b>	<b>−0.862</b>	<b>0.067</b>	<b>0.041</b>	<b>y</b>	<b>−1.621</b>	<b>0.088</b>	<b>0.027</b>	<b>y*</b>
Fractions	Aliphatic	0.037	−0.002	0.169		−0.053	0.013	0.123		−0.069	−0.006	0.189	
	Branched chain	0.000	−0.033	0.499		<b>0.063</b>	<b>0.045</b>	<b>0.063</b>	<b>n</b>	0.061	−0.008	0.196	
	Indolic	−0.006	−0.026	0.320		−0.003	−0.032	0.414		<b>0.038</b>	<b>0.043</b>	<b>0.067</b>	<b>n</b>
	Aromatic	<b>−0.027</b>	<b>0.07</b>	<b>0.039</b>	<b>y</b>	−0.015	−0.013	0.222		−0.040	0.019	0.107	
Diversity	Richness <i>S</i>	0.190	−0.022	0.286		−0.178	−0.027	0.329		0.214	−0.03	0.378	
	Shannon's <i>H</i>	−0.038	−0.018	0.253		−0.003	−0.033	0.481		−0.002	−0.033	0.494	
	CCI	<b>−0.170</b>	<b>0.053</b>	<b>0.054</b>	<b>y</b>	−0.089	−0.017	0.247		−0.049	−0.032	0.413	

Phylogenetic generalized least-squares (PGLS) models were estimated with the PGLS function from the R package CAPER (Orme *et al.*, 2013). Two species (*Streptanthus tortuosus*, *Caulanthus amplexicaulis*) have populations on and off serpentine, which differ in soil chemical attributes (Cacho & Strauss, 2014) and were kept separate for analyses. Soil nutrient variables were log-transformed. C3 and C4 represent carbon chain length of aliphatic glucosinolates; CCI, chemical complexity index, after Becerra *et al.* (2009). *P*-values were divided by 2 to adjust for one-tailed test, and also adjusted for multiple comparisons as follows: totals (five comparisons), aliphatics (three comparisons), fractions (four comparisons), diversity (three comparisons). The RAH column indicates support (y) or lack thereof (n) for the resource availability hypothesis (RAH). Significant relationships (at  $\alpha < 0.1$ ) are highlighted in bold, and those relationships that are significant after adjusting *P*-values for multiple comparisons are indicated with an asterisk (\*). The relationship of sum indolic with K (•) is driven by a single point and is thus not highlighted.

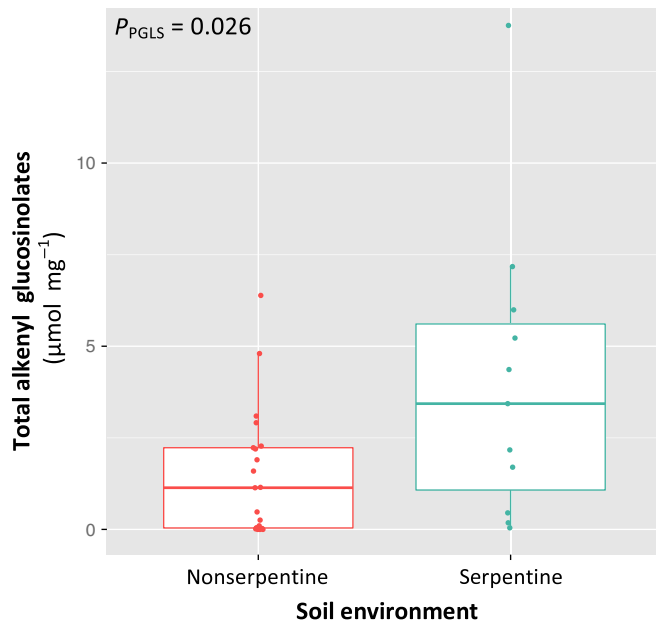
Strauss, 2007; Müller, 2009) across habitats. That said, studies using common-garden approaches also find low to moderate phylogenetic signal in secondary compounds (Agrawal *et al.*, 2009; Johnson *et al.*, 2009; Karinho-Betancourt *et al.*, 2015), and for a subset of our data ( $n = 15$ ) we found a significant, moderate correlation between total glucosinolates measured in the field and in the glasshouse. *Oenothera*'s flavonoids (Johnson *et al.*, 2014), *Quercus*' tannins (Pearse & Hipp, 2009), *Inga*'s phenolics (Kursar *et al.*, 2009), *Datura*'s alkaloids (Karinho-Betancourt *et al.*, 2015), and *Bursera*'s terpenes (Becerra *et al.*, 2009) also have weak phylogenetic signal. In *Asclepias*, phylogenetic signal is low for phenolics, moderate for flavonoids and coumaric and caffeic acid derivatives, and stronger for cardenolides (Agrawal *et al.*, 2009). Thus, it seems likely that glucosinolates in *Streptanthus* reflect a general pattern of weak phylogenetic signal in total production of secondary compounds.

Differential allocation to specific glucosinolates has stronger phylogenetic signal, specifically the fractions of aliphatics ( $K = 0.8$ ,  $P = 0.007$ ) and branched-chain glucosinolates ( $K = 0.9$ ,  $P = 0.04$ ). These two classes show a 'mirrored' pattern of variation across our phylogeny (i.e. when one is high, the other is low), suggesting tradeoffs in their production (Figs S1, S2). Recently, a gene polymorphism that creates this same 'mirrored' pattern between methionine-derived aliphatics and branched-chain glucosinolates was cloned in the mustard *Boechera* (Schrantz *et al.*, 2007). Alkenyl and C4 aliphatics also have moderate phylogenetic signal, and their production has been linked to two genes in *Arabidopsis*: *GS-Elong* (Magrath *et al.*, 1994; Kroymann *et al.*, 2003) and the *AOP* locus (Kliebenstein *et al.*, 2001, 2005; Gigolashvili *et al.*, 2007; Neal *et al.*, 2010). Both genes have been

associated with resistance to a range of lepidopteran, aphid, avian, mollusk and beetle pests, under both laboratory conditions and field conditions (Giamoustaris & Mithen, 1995; Kroymann & Mitchell-Olds, 2005; Hansen *et al.*, 2008; Züst *et al.*, 2012). In sum, phylogenetic signal is weak for total glucosinolate production in *Streptanthus*, and stronger for proportions of glucosinolate classes.

Ehrlich & Raven (1964) hypothesized that plant–herbivore coevolutionary arms races drove speciation and could explain the great diversity of plant secondary chemistry, plants, and their insect herbivores (defense-escalation-speciation hypothesis). A prediction of this hypothesis is that defensive compounds should become more abundant, more toxic, or more complex over evolutionary time. We found no evidence for escalation in glucosinolate complexity, but rather the contrary: a general pattern of evolutionary decline in glucosinolate richness, diversity, and chemical complexity.

Evolutionary chemical de-escalation has also been documented in cardenolides, latex, and trichomes of *Asclepias* (Agrawal & Fishbein, 2008; Agrawal *et al.*, 2009). These declines, and the ones we report in *Streptanthus*, are in contrast to terpene evolution in *Bursera*, where both richness and complexity tend to increase over evolutionary time, but still at a slower rate than species accumulate (Becerra *et al.*, 2009). Agrawal & Fishbein (2008) found increases in tolerance to herbivory with declining chemical complexity. We have observed that many species of *Streptanthus* are resilient to heavy herbivory; the relationship between tolerance and chemical defense over evolutionary time, and possible contrasting patterns, is an avenue of future work.



**Fig. 3** Alkenyl glucosinolates are higher in *Streptanthus* s.l. inhabiting serpentine soils, taking phylogeny into account (adjusted for multiple comparisons,  $P_{\text{PGLS}} = 0.04$ ). PGLS models were implemented with the function `PGLS` from the R package `CAPER` (Orme *et al.*, 2013), and the maximum credibility tree of a 50 million generation Bayesian analysis (Cacho *et al.*, 2014). Boxplot height corresponds to the first and third quartiles (Q1 and Q3), and the middle line to the median. Ranges are estimated with the '1.5 rule' (bounded by  $Q1 - 1.5 \times \text{interquartile range}$  (IQR) and  $Q3 + 1.5 \times \text{IQR}$ , where  $\text{IQR} = Q3 - Q1$ ). For estimates of this and other glucosinolates' relationships with microhabitat bareness or serpentine soil, see Table 5. Alkenyls are low-molecular-weight glucosinolates that are also highly volatile and that have the potential to function as host-finding attractants by herbivores specialists in Brassicaceae (Chew & Renwick, 1994), and could thus play roles in mediating higher trophic level responses against herbivory in serpentine habitats, where herbivore pressure might be high as a result of higher apparency (Strauss & Cacho, 2013).

A contributor to decreased chemical diversity in *Streptanthus* has been the increase in the dominance of aliphatics. The fraction of aliphatics has phylogenetic signal and increases over evolutionary time ( $P \leq 0.001$ ; Fig. 1). Trends towards specialization in certain compounds have been documented in other systems. In *Asclepias*, chemical diversity was positively correlated with cardenolide production, which traded off with fewer polar compounds (Rasmann & Agrawal, 2011). In *Bursera*, the evolutionary rate at which chemical diversity is generated has slowed down (Becerra *et al.*, 2009), suggesting either an increasing dominance of specific terpenes or an unlikely saturation of the terpenoid chemical pathway. Species specializing in different defense compounds could yield a pattern of decline in species' chemical diversity, as in *Bursera*, without an ensuing loss of a group's overall chemical richness.

Aliphatics have been implicated in deterring generalist herbivores and, in some cases, attracting specialists (Kroymann *et al.*, 2003), and could also play important roles in the occupation of increasingly bare environments, which are drier (Sambatti & Rice, 2006) and are associated with higher rates of herbivory in some *Streptanthus* species (Strauss & Cacho, 2013).

Glucosinolate biosynthesis in *Brassica* increases up to 62% under combined drought and root herbivory treatments (Tariq *et al.*, 2013), and many of these glucosinolates are aliphatics (e.g. 2-hydroxy-3-butenyl, an alkenyl). In another species of *Brassica*, top-soil drying led to an increase in aliphatics but not aromatic or indolic glucosinolates (Tong *et al.*, 2014). Thus, an evolutionary increase in the dominance of aliphatics might also be associated with the escalation in the bareness of microhabitats occupied by plants, beyond interactions with herbivores.

Another explanation of decreasing chemical diversity could emerge from tradeoffs with other forms of defense (e.g. the opposing patterns of tolerance and cardenolide production in *Asclepias*; Agrawal & Fishbein, 2008). Nickel hyperaccumulation in *Streptanthus polygaloides* serves as an elemental defense against herbivory (Boyd *et al.*, 1994; Jhee *et al.*, 2005). *S. polygaloides* also has the lowest glucosinolate production in the group (Fig. S1), suggesting a tradeoff. Reduction in glucosinolates in favor of other defenses might confound observed macroevolutionary patterns (Rudgers *et al.*, 2004; Agrawal & Fishbein, 2008), but excluding *S. polygaloides* from our analyses did not alter any of our results. *Streptanthus* exhibits a large variety of defensive traits not associated with glucosinolates, such as trichomes and resinous glands (*Streptanthus glandulosus*), or coarse leaves with thick cuticles (*Streptanthus morrisonii*). At least five *Streptanthus* species use a crypsis defense through leaf-soil matching (Strauss & Cacho, 2013); others produce orange callosities that have been shown, experimentally, to deter cannibalistic pierid-butterfly oviposition in *Streptanthus* through mimicking pierid eggs (Shapiro, 1981b). Thus, glucosinolates might not reflect all of the responses to selection imposed by herbivores, especially if the most important ones are specialists using secondary compounds as cues, or even to their advantage (Agrawal & Fishbein, 2008; Chaplin-Kramer *et al.*, 2011; Kos *et al.*, 2012).

### Macroevolutionary test of the RAH

The RAH predicts that plants inhabiting environments where tissue replacement is costly should invest more in defense (Coley *et al.*, 1985) and has been implicated in the processes driving edaphic specialization (Janzen, 1974; Fine *et al.*, 2004). Others have included water availability as a limiting resource when testing the RAH predictions (Cunningham *et al.*, 1999). For *Streptanthus*, several types of growing environment may incur high costs of tissue replacement. Among these are serpentine soils, which at our sites are low in Ca : Mg ratios, P and K, and high in toxic metals (Kruckeberg, 1954; Brady *et al.*, 2005; Cacho & Strauss, 2014). More generally, *Streptanthus* species grow in barer, drier, unvegetated habitats, which may provide challenges for plant life, such as higher exposure to UV radiation, apparency to enemies, and herbivore pressure. Also, the occupation of bare environments could have been a precursor to serpentine use in *Streptanthus* (Cacho & Strauss, 2014).

We found, in contrast to predictions of RAH, that total glucosinolate production was not related to any measure of resource availability or other stress. In support of the RAH, we found that:

**Table 5** Relationship between glucosinolate chemical defense and field microhabitat bareness where plants grow and serpentine soil in *Streptanthus* s.l

Variable kind	Variable	Microhabitat bareness				Serpentine soil					
		Estimate	Adj $r^2$	$P$	Adj $P$	RAH	Estimate	Adj $r^2$	$P$	Adj $P$	RAH
Total (main classes)	Total	1.622	−0.022	0.282	1.409		1.300	0.017	0.113	0.565	
	Aliphatic	2.382	−0.005	0.181	0.903		1.605	0.054	0.053	0.265	
	Branched chain	−1.235	0.005	0.147	0.733		−0.312	−0.017	0.247	1.234	
	Indolic	0.323	−0.001	0.165	0.823		−0.032	−0.031	0.402	2.011	
	Aromatic	0.170	−0.018	0.252	1.260		0.101	0.003	0.152	0.759	
Aliphatic subclasses	C3	2.597	0.012	0.124	0.373		0.847	−0.001	0.166	0.497	
	C4	−0.080	−0.033	0.477	1.431		<b>0.967</b>	<b>0.081</b>	<b>0.031</b>	<b>0.094</b>	y*
	Alkenyl	1.880	−0.016	0.242	0.727		<b>2.221</b>	<b>0.126</b>	<b>0.013</b>	<b>0.040</b>	y*
Fractions	Aliphatic	0.381	0.051	0.056	0.225		0.078	−0.01	0.204	0.815	
	Branched chain	− <b>0.406</b>	<b>0.084</b>	<b>0.030</b>	0.119	n	−0.067	−0.012	0.216	0.865	
	Indolic	0.017	−0.032	0.416	1.662		0.001	−0.033	0.486	1.946	
	Aromatic	0.033	−0.03	0.372	1.487		−0.01	−0.031	0.403	1.613	
Diversity	Richness $S$	<b>4.685</b>	<b>0.134</b>	<b>0.011</b>	<b>0.045</b>	y*	0.437	−0.024	0.298	1.194	
	Shannon $H$	0.034	−0.033	0.463	1.852		0.047	−0.029	0.368	1.472	
	CCI	0.314	−0.026	0.324	0.912		0.165	−0.020	0.207	0.830	

Phylogenetic generalized least-squares (PGLS) models were estimated using the `PGLS` function from the package `CAPER` (Orme *et al.*, 2013) in R (R Core Team, 2014), and the maximum credibility tree of a Bayesian 50 million generation analysis (Cacho & Strauss, 2014). Two species (*Streptanthus tortuosus*, *Caulanthus amplexicaulis*) have populations on and off serpentine, which differ in bareness (Cacho & Strauss, 2014) and were kept separate for analyses. Bareness values were arcsine-square-root-transformed. Variables C3 and C4 represent carbon chain length of aliphatic glucosinolate compounds. CCI, chemical complexity index (see text).  $P$ -values were divided by 2 to adjust for one-tailed test, and also adjusted for multiple comparisons as follows: totals (five comparisons), aliphatics (three comparisons), fractions (four comparisons), diversity (three comparisons). The RAH column indicates support (y) or lack thereof (n) for the resource availability hypothesis (RAH). Significant relationships (at  $\alpha < 0.1$ ) are in bold, and those relationships that are significant after adjusting  $P$ -values for multiple comparisons are indicated with an asterisk (\*).

glucosinolate richness increases with habitat bareness; the amounts of C3 and alkenyl aliphatics are higher in environments where all three nutrients measured ( $\text{NO}_3\text{N}$ , P, K) are low; and a general preponderance of negative relationships between glucosinolates and nutrients. Ten out of 14 significant correlations (and both that remain significant after correcting for multiple comparisons) are in the direction predicted by the RAH. Where we found positive relationships, they mostly involved branched-chain glucosinolates, which trade off with aliphatics.

Possessing a chemical arsenal with a greater number of compounds may represent an increased investment in defense, if more enzymatic reactions are required to overcome it (Li *et al.*, 2007). Chemical complexity was negatively related to nitrates, and glucosinolate richness was significantly related to habitat bareness, with more compounds favored in more bare habitats. Müller (2009) showed that more diverse combinations of glucosinolates conferred defense against a greater suite of enemies. Also, a more diverse glucosinolate production may be implicated in attracting herbivore predators (Chew & Renwick, 1994; De Vos *et al.*, 2008; Chaplin-Kramer *et al.*, 2011) or in allelopathy against plant competitors (Lankau, 2011, 2012).

Besides their direct and indirect roles in defense, glucosinolates could play additional roles in adaptation to bare environments. Our multivariate partitioning of variance and NMDS projections reveals that glucosinolate profiles vary with microhabitat bareness rather than with serpentine use or nutrients, contrary to RAH predictions. In *Brassica* and *Boechera*, growth slows down under drought conditions (Radovich *et al.*, 2005; Haugen *et al.*, 2008), and in *Brassica*, glucosinolate biosynthesis also increases

(Radovich *et al.*, 2005; Schreiner *et al.*, 2009; Tariq *et al.*, 2013). The effects of drought and root herbivory treatments in glucosinolates of *Brassica oleracea* were compound- and treatment-dependent (Tariq *et al.*, 2013): herbivory and drought had synergistic effects in the increase of 2-hydroxy-3-butenyl (aliphatic/alkenyl) and 4-methoxy-indol-3-ylmethyl (indole/4MO-I3M), while 4-methylsulfinylbutyl (aliphatic/nonalkenyl) and allyl (aliphatic/alkenyl) were at their lowest under high and medium drought conditions (Tariq *et al.*, 2013). Variation in individual glucosinolates' response to herbivory and water limitation were also observed in *Boechera* (Haugen *et al.*, 2008), and we already mentioned aliphatic-specific responses to drought (Tong *et al.*, 2014). Our own experiments have shown that rates of attack on *Streptanthus* are higher in bare habitats, where other defense strategies might be favored (Strauss & Cacho, 2013). Explicit experimental approaches will be necessary to evaluate which functions of glucosinolates are under the strongest selection in different environmental contexts across this group.

### Concluding remarks

We find weak to moderate phylogenetic signal in glucosinolate classes synthesized by plants, and a fairly strong evolutionary pattern of increased dominance of aliphatic glucosinolates. Support for the RAH is partial with respect to soil nutrients, and lacking in relation to serpentine use. The number of glucosinolate compounds increases with the bareness of microhabitats occupied by plants; bareness has been related to both higher herbivory rates (Strauss & Cacho, 2013) and increased drought pressure. Habitat



bareness is highly conserved across this group and escalates over evolutionary time; it may be an underappreciated source of selection on many plant traits, including glucosinolates.

The RAH predicts increased defense in harsh environments, and environments occupied by streptanthoids have been increasingly more bare over time. Likewise, the defense-escalation hypothesis also predicts an increase of defense over evolutionary time. Our data reveal de-escalation trends in richness and complexity of glucosinolate defense, and thus limited support for the defense-escalation hypothesis as it pertains to glucosinolates and under the mechanisms proposed by Ehrlich & Raven (1964). The solution to this conundrum might lie in alternative strategies of defense employed by streptanthoids beyond glucosinolates. *Streptanthus* has many specialist herbivores that could have driven deceit defenses (e.g. egg-mimicry, crypsis) not involving glucosinolates. We may need a more holistic approach to evolution of defense to confidently reject escalation. In summary, our macroevolutionary approach attempts to understand and test hypotheses about the evolution of plant defense in light of the range of natural habitats occupied by a whole clade.

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## Supporting Information

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

**Fig. S1** Glucosinolate totals and amounts by classes are evolutionary labile.

**Fig. S2** Glucosinolate fractions by classes have stronger phylogenetic signal.

**Fig. S3** NMDS plots of glucosinolate profiles.

**Table S1** Thirty-five glucosinolate compounds in *Streptanthus* s.l.

**Table S2** Populations included in this study with data for all, glucosinolates, soils, and bareness

**Table S3** Phylogenetic signal in glucosinolate individual compounds

**Table S4** Field microhabitat bareness is correlated with glucosinolate profiles according to multivariate partitioning of variance analyses

**Table S5** Coefficients of a discriminant function that accurately classifies samples according to soil type (serpentine/nonserpentine)

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