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A test of elemental defence against slugs by Ni in hyperaccumulator and non-hyperaccumulator *Streptanthus* species

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Summary. Tissues of most plant species contain < 10 µg Ni g^{-1} but Ni hyperaccumulators contain more than 1000 µg Ni g^{-1}. Hyperaccumulated Ni can defend plants from some herbivores but the defensive role of lesser Ni concentrations is little explored. We raised five species of *Streptanthus* (Brassicaceae) native to ultramafic soils, one of which (*S. polygaloides*) is a Ni hyperaccumulator whereas the others are simply Ni-tolerant, on Ni-amended and unamended greenhouse soils to create plants differing in Ni concentrations. On high-Ni soil, leaves of the hyperaccumulator contained 3800 µg Ni g^{-1} whereas leaves of non-hyperaccumulator species contained 41–64 µg Ni g^{-1}. Plants of all species grown on low-Ni soils had < 14 µg Ni g^{-1}. Slugs (*Limax maximus*) were fed plant material in no-choice tests over a 50-day period and survival and mass changes were recorded. All slugs fed high-Ni leaves of the hyperaccumulator species died within 21 d. Slugs fed high-Ni leaves of the other species did not differ significantly in survival or mass change from those fed low-Ni leaves. In choice tests, slugs (*Lehmannia valentiana*) offered both high- and low-Ni *S. polygaloides* leaves did little damage to high-Ni leaves. We conclude that hyperacumulated Ni can defend *S. polygaloides* from slug herbivory via both toxicity and deterrence, but these defensive effects do not extend to *Streptanthus* species containing < 70 µg Ni g^{-1}.

Key words. Accumulator – elemental defences – heavy metals – herbivory – hyperaccumulation – nickel

Introduction

The elemental concentration of plant tissues can vary widely among species (Reeves & Baker 2000). The "normal" range of concentrations can be contrasted with accumulation (unusually elevated concentration) and with hyperaccumulation (extremely elevated levels). For Ni, Reeves & Baker (2000) define the normal range of plant concentrations as 1–10 µg Ni g^{-1}, with accumulation ranging from 100–1000 µg Ni g^{-1} and hyperaccumulation defined as > 1000 µg g^{-1}. Nickel is the element most often hyperaccumulated by plants: about 75 % of all hyperaccumulators are Ni hyperaccumulators (Reeves & Baker 2000).

A number of hypotheses have been suggested regarding the selective value of hyperaccumulation (Boyd & Martens 1992). For Ni, plant defence against herbivores has been explored by a number of studies (Boyd 1998; 2004). In general, hyperaccumulator concentrations of Ni are effective against folivores (Boyd & Martens 1994; Martens & Boyd 1994; Boyd & Moar 1999; Boyd *et al*. 2002; Jhee 2004), rhizovores (Jhee 2004) and some cell-disrupting herbivores (Jhee 2004), but not vascular tissue-feeding insects or other cell-disruptors (Boyd & Martens 1999; Jhee 2004). Herbivorous terrestrial mollusks can have large impacts on plants (*e.g*., Rodriguez & Brown 1998; Barker 2002; Frank 2003) and slug herbivory may limit the ranges of some species (*e.g*., Bruelheide & Scheidel 1999). Prior studies have explored defensive effects of Zn against snails (Huitson & Macnair 2003) and slugs (Pollard & Baker 1997), but only one study of defensive effects of Ni has been done using a molluscan herbivore, the snail *Helix aspersa* Müller (Mollusca: Pulmonidae) (Boyd *et al*. 2002). One purpose of our study is to explore the defensive effect of hyperaccumulated Ni on slugs.

Another question deserving further study is whether the defensive benefits of metals extend to species with elevated (but not hyperaccumulator) Ni levels (Boyd 2004). This idea has rarely been tested. Recent work using the insect folivore *Plutella xylostella* (Lepidoptera: Plutellidae) fed artificial diet amended with metals showed that minimum toxic levels of eight metals hyperaccumulated by plants (Cd, Co, Cr, Cu, Mn, Ni, Pb and Zn) were in the ranges found in accumulator plant species (Coleman *et al*. 2005). Five metals (Cd, Mn, Ni, Pb and Zn) were toxic to *P. xylostella* at concentrations below accumulator levels, Cd and Pb were toxic at concentrations near those of normal plants and Zn was toxic at a concentration within the normal plant range (Coleman *et al*. 2005). Thus, metals may have protective effects at accumulator or lesser concentrations. Our study tested the defensive effect of elevated Ni concentrations by using four species of *Streptanthus* (Brassicaceae) that grow on ultramafic soils and thus can contain Ni levels above those of normal plants (Kruckeberg & Reeves 1995). We also used a Ni hyperaccumulator species, *S. polygaloides*, that has been the focus of previous research on the defensive effects of hyperaccumulated Ni (*e.g*., Boyd & Moar 1999; Martens & Boyd 1994, 2002). We raised plants of all five species on both high-Ni and low-Ni soils to create plants with relatively high and low Ni concentrations and fed leaves to slugs to determine if their Ni levels had negative effects.

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The specific objectives of our study were to determine: 1) if leaves of the Ni hyperaccumulator *S. polygaloides* were toxic to slugs when they contained hyperaccumulator levels of Ni; 2) if leaves of species of *Streptanthus* containing elevated but not hyperaccumulator quantities of Ni caused toxic or sublethal effects to slugs; and 3) if slugs could discriminate between high- and low-Ni leaves of *S. polygaloides* plants in choice tests.

Materials and Methods

Study organisms (plants): Five species of *Streptanthus* were used. One (*Streptanthus polygaloides*) is the sole Ni hyperaccumulator in the genus (Reeves *et al*. 1981; Kruckeberg & Reeves 1995). It is an ultramafic soil endemic found on the western slope of California's Sierra Nevada (Hickman 1993). All populations investigated hyperaccumulate Ni in all parts of the plant (Reeves *et al.* 1981). Seeds for our experiment were obtained from populations growing in the Red Hills of Tuolumne County, approximating sample 6737 of Kruckeberg & Reeves (1995), and from an ultramafic area on the eastern shore of Big Pine Reservoir on the Kings River in Fresno County.

The other four *Streptanthus* species do not hyperaccumulate Ni in the wild (Kruckeberg & Reeves 1995) but three of them are also usually restricted to ultramafic soils. *Streptanthus breweri* is typically found on ultramafic barrens in the coastal mountains of California (Hickman 1993). The population from which we collected seeds is in Napa County at Turtle Rock (Shapiro 1981) and corresponds to sample 6742 of Kruckeberg & Reeves (1995). *Streptanthus insignis* is found in the southern coast ranges of California (Hickman 1993). Seeds of *S. insignis* were collected from a population growing in San Benito County about 10 km west of Panoche Pass (Kruckeberg 1984). *Streptanthus albidus* is restricted to the central coastal mountains of California (Hickman 1993). Seeds were collected from a population in Santa Clara County from an ultramafic grassland about 2 km east of US Highway 101 on Metcalf Road (Kruckeberg 1984). The last species, *S. tortuosus*, can be found both on and off of ultramafic soils (Hickman 1993). Seeds used for these experiments were collected from an ultramafic area along Washington Road in Nevada County. This site corresponds to sample 6732 of Kruckeberg & Reeves (1995) and was the site of a field study of herbivory on high- and low-Ni *S. polygaloides* by Martens & Boyd (2002).

Experimental soils that differed in Ni concentration were used to generate high- and low-Ni plants of each species. Both soils were a standard greenhouse potting medium (ProMix: Premier Horticulture, Red Hill, Pennsylvania, USA), but the high-Ni soil was amended to *ca.* 800 mg Ni kg^{-1} by adding anydrous $NiCl_2$ (Fisher Chemicals: Fairlawn, New Jersey, USA) to the soil. Low-Ni soil was unamended Pro-Mix. Small pots (20 X 20 cm square, 10 cm deep) were filled with soil, topped with a layer of perlite, and seeds sown. Plants were grown in a glasshouse under ambient light at *ca*. 20 °C and watered twice daily. Several sets of plants, sown 2–3 weeks apart in February and March 2002, were grown to provide a continuous supply of leaves to feed to slugs during the no-choice feeding trial (described below).

Study organisms (herbivores): Two introduced slug species, both belonging to the Limacidae (Mollusca), were used for feeding experiments. A no-choice feeding experiment used *Limax maximus* Linnaeus, a species native to Europe and Asia Minor (Grewal *et al*. 2003). These slugs were obtained from a biological supply company that collected them from a non-ultramafic area in North Carolina, USA in March 2002. They were maintained upon lettuce, *Lactuca sativa* L. (Asteraceae), for several days before the no-choice experiment began.

The second slug species, used for choice experiments using high- and low-Ni *S. polygaloides*, was *Lehmannia valentiana* (de Ferrusac), a species native to Europe (Barker 1999). Like most slugs (Barker 2002), it is a generalist feeder on a variety of plant species. In April 2003, specimens of *Lehmannia valentiana* were collected from yards of homes in Auburn, Lee County, Alabama, USA. Slugs were captured during a 4-day period and kept without food in containers with moistened paper towels until used for choice experiments.

Plant elemental analysis: Aboveground material of each species X soil combination was harvested periodically for analysis of elemental composition. Over the 2 month period of the no-choice feeding trial, plant samples were collected a total of six times. Because of low biomass for some samples, samples were combined to create four replicates for each species X soil combination. Plant samples were dried for at least 3 days at 65 °C, ground with mortar and pestle, dry-ashed at 485 °C, further oxidized in 1 M HNO3, and the residues were re-dissolved in 1 M HCl. Element concentrations were determined using an inductively coupled argon plasma (ICP-AE) spectrophotometer (SPECTRO CIROS CCD: Kleve, Germany). Carbon and N % were measured by combusting 0.5 g plant samples using a LECO CN-2000 Analyzer (LECO Corporation: St. Joseph, MO, USA). Sulfur % was determined by combusting 0.5 g plant samples in a LECO SC-432 Analyzer (LECO Corporation: St. Joseph, MO, USA).

No-choice feeding trial: This experiment was conducted on a laboratory bench at room temperature (*ca*. 22 °C). Plastic 150 ml containers were used. About 2 cm of wetted sand was placed at the bottom of each container to increase humidity and a piece of wetted filter paper (Whatman #40) was placed on top of the sand. Slugs were randomly assigned to each of 110 containers.

Slugs were fed leaves of each species from plants grown on either high- or low-Ni soil. There were 10 replications of each experimental treatment and we included 10 slugs that were not fed. The latter treatment was included so that we could compare mortality patterns of slugs offered leaves against those of slugs that did not feed. Fresh leaves were added to containers every few days and leftover leaves removed. At weekly intervals for 7 weeks, each surviving slug was removed and weighed, its container cleaned and the filter paper replaced. After 3 weeks, the experiment was ended for slugs fed low-Ni *S. polygaloides* leaves and for those not fed at all because all slugs fed high-Ni leaves had died by that time.

Survival analyses were used to determine effects of leaf Ni levels on slug survival. Data were analyzed using StatView (Abacus Concepts 1998) utilizing the Kaplan-Meier estimate with treatment significance determined by the Peto-Peto-Wilcoxon test at $\alpha \leq 0.05$ (Abacus Concepts 1998). Survival curves of slugs fed high- or low-Ni *S. polygaloides* leaves were compared to each other and also to that of slugs that were not fed. Separate survival analyses contrasted survival of slugs fed high- vs. low-Ni leaves of *S. breweri*, *S. insignis* and *S. tortuosus*. Survival analysis was not done for slugs fed *S. albidus* leaves because no mortality occurred. Mass data for slugs fed species other than *S. polygaloides* were analyzed with repeated measures analysis of variance (ANOVA) using species and treatment as main effects factors and including the species X treatment interaction term.

S. polygaloides choice test: Because high-Ni *S. polygaloides* was toxic to slugs (see Results), we used choice tests to determine whether slugs avoided consuming hyperaccumulating plants. Two tests were conducted with a total of 30 *Lehmannia valentiana*. Each slug was placed into a plastic container (150 ml containers, described above) on a laboratory bench at room temperature (*ca*. 22 °C). Into each container was placed a pair (one high-Ni and one low-Ni) of *S. polygaloides* plants grown from seeds collected in Fresno County. Each plant was cut to about 10 cm length and matched for size. Plants were weighed before they were placed into the containers. Plants of a pair were placed on opposite sides of the container and the cut stem of each was inserted into the wet sand at the bottom of the container to help maintain plant freshness. After plants were placed into containers we were unable to distinguish high-Ni from low-Ni plants. Slugs were allowed to feed for 10 days. Plants were then removed from the containers and the degree of herbivore damage to each compared. Both plants were tested for Ni concentration using filter paper treated with dimethylglyoxime (DMG) and the Ni concentration of the plant less damaged was recorded. DMG paper turns pink when it absorbs juice from Ni hyperaccumulating plants (Reeves 1992). Thus, herbivore

Table 1 Two-way ANOVA results for elemental analysis of aboveground biomass of *Streptanthus* species grown for the no-choice feeding trial. Degrees of freedom for *F*-values are noted parenthetically in column headings

	ANOVA Factor/Interaction					
	Nickel addition		*Streptanthus* species		Nickel * Species	
Element/Value	*F* (1,30)	*P*	*F* (4,30)	*P*	*F* (4,30)	*P*
C (%)	12	0.0018	16	< 0.0001	0.090	0.99
Ca (μg g^{-1})	7.3	0.011	70	< 0.0001	0.64	0.64
Cu (μg g^{-1})	0.95	0.34	8.7	< 0.0001	0.72	0.58
Fe (μg g^{-1})	0.53	0.47	49	< 0.0001	0.40	0.81
K (%)	0.77	0.39	10	< 0.0001	0.49	0.74
Mg (%)	11	0.0020	36	< 0.0001	1.8	0.15
Mn (μg g^{-1})	7.8	0.0090	6.6	0.0006	1.2	0.34
N (%)	0.0030	0.96	8.7	< 0.0001	0.58	0.68
N/C (%)	0.052	0.82	9.0	< 0.0001	0.54	0.71
Ni (μg g^{-1})	73	< 0.0001	65	< 0.0001	64	< 0.0001
P (%)	0.83	0.37	60	< 0.0001	0.61	0.66
S (%)	0.052	0.82	18	< 0.0001	5.2	0.0026
Zn (μg g^{-1})	6.6	0.016	43	< 0.0001	2.4	0.070

damage was assessed before the experimenter knew the Ni level of each plant. The experiment was repeated once after containers were cleaned, using the same slugs and the same procedures as for the first experimental run.

Initial plant masses were compared (*t*-test, $\alpha \leq 0.05$) to ensure that plant sizes of high- and low-Ni plants were comparable in each run. Choice data were analyzed using contingency table analysis to test if high- or low-Ni plants were damaged more greatly than predicted by chance. Slugs that did not feed on either plant during the experiment were excluded from choice data analyses. Samples of plant material used for this experiment (three each from high- and low-Ni plants) were dried for several days at 65 °C and analyzed as above for elemental concentrations.

Results

Plant elemental analysis: Elemental analyses revealed many differences due to both species and soil treatment (Table 1). Plant Ni concentration varied more than any of the six parameters significantly affected by Ni treatment of soil. Plants grown on low Ni soils contained from 0.68 μg Ni g^{-1} for *S. tortuosus* to 13 μg Ni g^{-1} (for *S. polygaloides*) (Table 2), whereas those grown on high Ni soils contained 41–64 μg Ni g^{-1} for the non-hyperaccumulator species and 3800 μg Ni g^{-1} for *S. polygaloides*. Nickel addition also significantly affected C, Ca, Mg, Mn and Zn values (Table 1). Concentrations of all these elements increased in plants grown on Ni-amended soils except for C, which decreased (Table 2).

The species factor significantly varied for every element or value analyzed (Table 1). Only for Ni, however, did *S. polygaloides* differ significantly from all other species, yet the other species did not differ from each another (Table 2). *Streptanthus polygaloides* also contained significantly more Fe, K, Mn, P and Zn than the other *Streptanthus* species (Table 2).

Significant Ni X species interactions were comparatively scarce: only two were detected (for Ni and S). In the case of Ni, *S. polygaloides* on high-Ni soil had 290–fold more Ni than on low-Ni soil, whereas other species showed 13–fold (*S. insignis*) to 60–fold (*S. tortuosus*) differences (Table 2). For S, only high-Ni *S. polygaloides* contained less S (almost 50 % less) than plants grown on low-Ni soil whereas other species showed the opposite trend (Table 2).

No-choice feeding trial: *Limax maximus* slugs fed high-Ni *S. polygaloides* all died by day 21, whereas most fed low-Ni leaves were still alive at that time (Fig. 1). Even most slugs that were not fed were alive at day 21, indicating that slugs given high-Ni *S. polygaloides* leaves died due to toxic effects of their diet rather than because they refused to feed. Survival analysis showed that the survival curve for slugs fed high-Ni *S. polygaloides* significantly differed from that for slugs fed low-Ni *S. polygaloides* ($P = 0.044$) and for slugs not fed ($P = 0.0016$), whereas the latter two curves did not differ significantly from each other ($P = 0.41$).

Slug survival was comparatively high for all other treatments, ranging between 80 and 100 % after 50 days (Fig. 1). Survival analyses comparing slugs fed high- and low-Ni plants of *S. breweri, S. insignis* and *S. tortuosus* showed no significant difference due to soil Ni treatment ($P > 0.48$ for each comparison). No slugs fed either high- or low-Ni *S. albidus* leaves died during the experiment, precluding the need for statistical analysis.

We also analyzed slug mass changes using repeated measures ANOVA for those treatments for which no treatment (Ni) effect on survival was found because a treatment effect on slug mass could detect sublethal effects of high-Ni leaves. In general, slugs gained mass over time on all leaf Ni treatments and species (Fig. 2). In the repeated measures ANOVA results, significant effects of experimental factors are revealed by significant time X factor interactions. The ANOVA showed significant effects of plant species because the time X species interaction was significant (Wilk's Lambda: $F_{21,164} = 7.0$, $P < 0.0001$). Slug mass increased to different degrees for slugs fed different species (Fig. 2). For example, slugs fed *S. albidus* increased in mass more than those fed *S. breweri* (Fig. 2). Nickel treatment did not influence slug

Table 2 Elemental analysis of aboveground biomass of *Streptanthus* species grown for the no-choice feeding trial. Data are means (SE), $N = 4$. Letters underneath groups of Ni+ and Ni– means denote results of post-hoc comparisons of means for each species (Fisher's PLSD test, $\alpha < 0.05$). Species with the same letter do not differ significantly in concentrations for that element or value. *Streptanthus polygaloides* plants were raised from seeds collected from the Red Hills of Tuolumne County

	Streptanthus species														
	S. albidus			*S. breweri*			*S. insignis*			*S. polygaloides*			*S. tortuosus*		
Element or Value	Ni+		Ni–	Ni+		Ni–	Ni+		Ni–	Ni+		Ni–	Ni+		Ni–
C (%)	39 (0.65)	b	40 (0.28)	40 (0.35)	a	41 (0.39)	39 (0.37)	b	39 (0.25)	37 (0.14)	c	38 (0.39)	39 (0.44)	b	40 (0.083)
Ca ($\mu g\ g^{-1}$)	2.5 (0.044)	b	2.2 (0.12)	1.4 (0.11)	c	1.3 (0.13)	2.8 (0.080)	a	2.5 (0.20)	2.5 (0.086)	a,b	2.4 (0.12)	1.3 (0.13)	c	1.1 (0.037)
Cu ($\mu g\ g^{-1}$)	7.9 (0.45)	b	7.9 (0.72)	8.3 (1.1)	b	8.3 (0.82)	10 (2.1)	a	14 (2.2)	15 (2.0)	a	14 (1.3)	6.2 (2.0)	b	8.5 (0.40)
Fe ($\mu g\ g^{-1}$)	46 (4.0)	b	49 (1.9)	40 (2.8)	c	39 (3.5)	49 (2.1)	b	51 (1.3)	68 (4.8)	a	67 (2.3)	28 (1.8)	d	32 (0.91)
K (%)	2.9 (0.21)	b	2.9 (0.21)	2.3 (0.20)	c	2.3 (0.28)	2.7 (0.15)	b	2.9 (0.12)	3.1 (0.066)	a	3.5 (0.11)	2.4 (0.24)	c	2.4 (0.055)
Mg (%)	0.27 (0.039)	c	0.25 (0.009)	0.63 (0.055)	a	0.48 (0.053)	0.38 (0.019)	b	0.31 (0.02)	0.41 (0.023)	b	0.30 (0.014)	0.18 (0.046)	d	0.18 (0.008)
Mn ($\mu g\ g^{-1}$)	38 (1.2)	c	30 (3.4)	60 (6.8)	b	35 (2.7)	51 (2.7)	b	50 (11)	78 (9.7)	a	58 (11)	52 (3.8)	b	47 (4.6)
N (%)	2.6 (0.23)	a,b	2.7 (0.20)	2.3 (0.36)	b,c	2.4 (0.47)	3.2 (0.21)	a	3.0 (0.17)	2.9 (0.24)	a	3.4 (0.28)	2.0 (0.17)	c	1.7 (0.099)
N/C (%)	6.8 (0.71)	b,c	6.7 (0.55)	5.8 (0.97)	c	5.9 (1.2)	8.3 (0.62)	a,b	7.5 (0.48)	7.9 (0.64)	a	8.9 (0.80)	5.0 (0.48)	c	4.2 (0.25)
Ni ($\mu g\ g^{-1}$)	53 (6.2)	b	1.6 (0.10)	48 (2.5)	b	0.85 (0.10)	64 (7.1)	b	4.9 (2.2)	3800 (470)	a	13 (7.1)	41 (5.5)	b	0.68 (0.11)
P (%)	0.54 (0.030)	c	0.58 (0.031)	0.32 (0.019)	d	0.33 (0.031)	0.55 (0.009)	c	0.61 (0.025)	0.91 (0.033)	a	0.97 (0.029)	0.78 (0.11)	b	0.73 (0.020)
S (%)	0.49 (0.035)	c	0.39 (0.042)	0.54 (0.055)	b,c	0.49 (0.082)	0.60 (0.013)	b	0.59 (0.020)	0.35 (0.022)	b,c	0.67 (0.036)	1.0 (0.13)	a	0.81 (0.082)
Zn ($\mu g\ g^{-1}$)	240 (18)	c	200 (18)	240 (37)	c	180 (18)	250 (13)	c	220 (21)	830 (110)	a	580 (35)	370 (60)	b	380 (38)

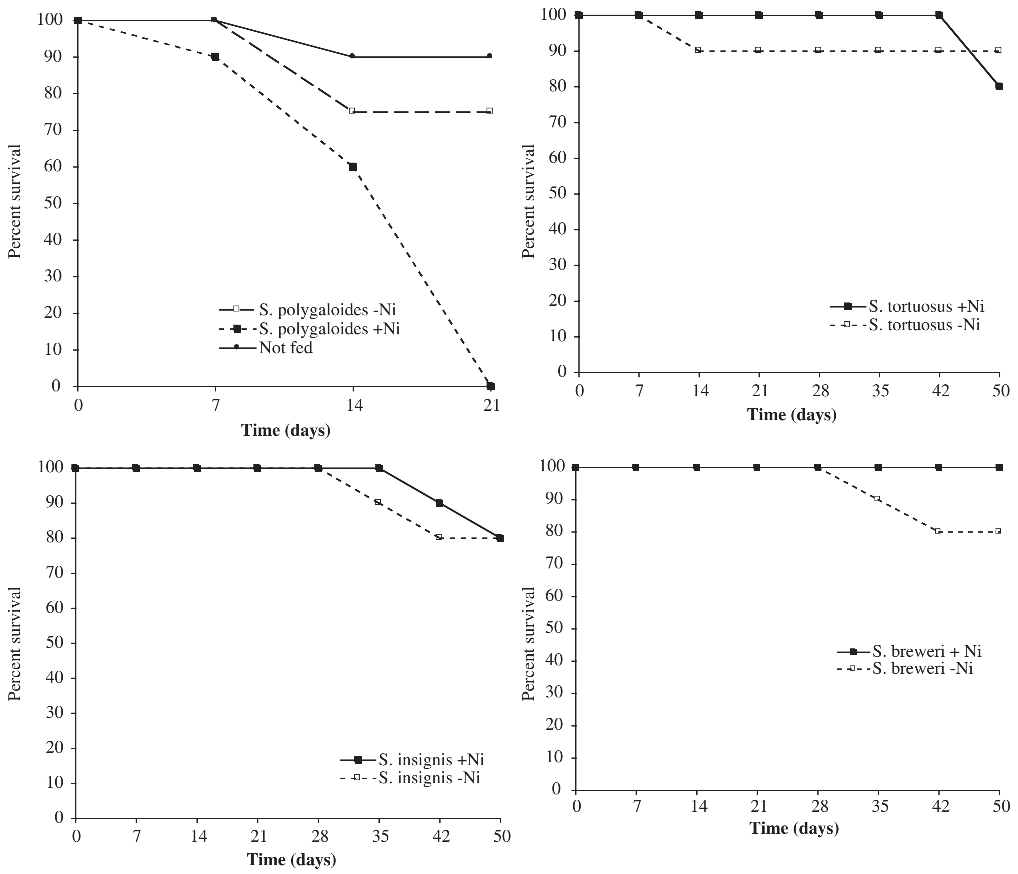

Fig. 1 Survival curves of *Limax maximus* fed high- or low-Ni leaves of *Streptanthus* species. Results from slugs that were not fed are graphed with those from slugs fed *S. polygaloides* leaves (top left). Results from slugs fed high-Ni or low-Ni *S. albidus* leaves are not shown since no slugs died while feeding on that species

mass, as the time X treatment interaction was not significant (Wilk's Lambda: $F_{7,57} = 0.26$, $P = 0.967$). The time X species X treatment interaction also was not significant (Wilk's Lambda: $F_{21,164} = 0.72$, $P = 0.512$), indicating a lack of a species X treatment interaction.

S. polygaloides choice test: High- and low-Ni plants were similar in fresh mass at the start of both runs of the choice test (Table 3). Slugs strongly preferred low-Ni plants in both runs (Table 3). In no case was there more damage to the high-Ni plant and, in almost all cases, no damage was observed to the high-Ni plant. Elemental analysis of plants used in the choice test showed that the largest difference measured was for plant Ni concentration, which was 72-fold greater for plants grown on high-Ni soil (Table 4). Significant differences were also detected for Ca, Fe, K and P, for which only Ca was greater for high-Ni plants (Table 4).

Discussion

Slugs fed *S. polygaloides* grown on high-Ni soil died, indicating that those plants were toxic. These slugs did not starve because their mortality was significantly greater than that of slugs that were not fed (Fig. 1). The cause of this toxic effect can be inferred from results of plant tissue analysis, but this is difficult because the plants varied considerably in elemental make-up. Both species differences as well as soil treatment contributed to this variation (Tables 1, 2). We argue that the very high Ni concentration in *S. polygaloides* grown on high-Ni soil was likely responsible for the increased mortality of slugs fed high-Ni *S. polygaloides* leaves. Of all the parameters measured in Table 2, the greatest difference between high- and low-Ni *S. polygaloides* was the extremely high Ni concentration of high-Ni *S. polygaloides*.

We conclude that our experiment shows hyperaccumulated Ni defends *S. polygaloides* against slugs. This result adds two slug species to the list of herbivores against which Ni hyperaccumulation has been shown to have defensive effects (Boyd 1998; Boyd 2004). Only one other mollusk (*Helix aspersa*) has been tested for defensive effects of hyperaccumulated Ni and Ni was effective in that case as well (Boyd *et al*. 2002). Jhee (2004) reported that herbivore feeding mode can influence the defensive effectiveness of hyperaccumulated Ni in *S. polygaloides*. He found that leaf-chewing folivores were susceptible to Ni whereas herbivores feeding on vascular tissues were unaffected. Both slug species we used are folivores. Thus our results reinforce the conclusion of Jhee (2004) that hyperaccumulated Ni is an effective elemental defence against herbivores of that feeding mode.

Our choice experiment also showed that hyperaccumulated Ni had strong effects on slug choice. Deterrence of herbivory is particularly beneficial to a plant if it can prevent feeding damage. Pollard & Baker (1997) reported that Zn

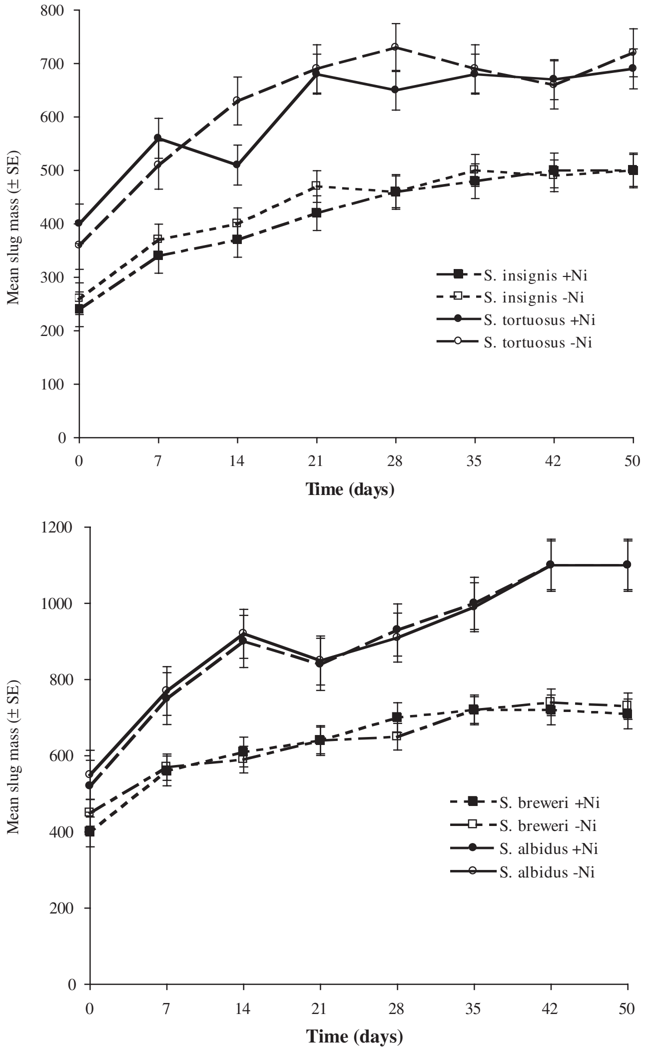

Fig. 2 Mean masses of *Limax maximus* fed high- or low-Ni leaves of *Streptanthus* species during the no-choice feeding experiment. Top: masses of slugs fed *S. insignis* or *S. tortuosus*. Bottom: masses of slugs fed *S. breweri* or *S. albidus*. Error bars are ± 1 SE

Table 3 Results of choice tests of *L. valentiana* provided high- and low-Ni *S. polygaloides* plants (each run used 30 slugs). Plant mass is mean fresh plant weight at the start of each run (SE in parentheses, $N = 30$). Preference is the percentage of replicates in each run for which more damage was done to the plant of that metal concentration. Preference percentages do not sum to 100% because some slugs did not feed on either plant. Test statistic reports results of *t*-tests for plant mass and contingency table analyses for preference data

Experiment and measure	Treatment (plant Ni level)			
	High-Ni	Low-Ni	Test statistic	*P*-value
Run 1:				
Mean plant mass (mg)	288 (14.0)	290 (14.0)	$t = 0.094$	0.926
Preference	0%	67%	$X^2 = 40$	< 0.0001
Run 2:				
Mean plant mass (mg)	165 (7.18)	160 (6.38)	$t = 0.489$	0.626
Preference	0%	70%	$X^2 = 42$	< 0.0001

Table 4 Elemental analysis of *Streptanthus polygaloides* plants raised from seeds collected from the Fresno County population and used for choice experiments. Results are means (SE), $N = 3$, along with results of *t*-tests analyzing each element for differences between high- and low-Ni plants

Element (units)	High-Ni	Low-Ni	*t*-value	*P*-value
Ca (μg g^{-1})	1.3 (0.017)	1.1 (0.006)	10	0.0005
Cu (μg g^{-1})	18 (0.15)	22 (2.4)	1.5	0.21
Fe (μg g^{-1})	31 (0.29)	45 (3.0)	4.5	0.011
K (%)	2.1 (0.033)	2.7 (0.015)	17	< 0.0001
Mg (%)	0.31 (0.006)	0.29 (0.009)	2.2	0.091
Mn (μg g^{-1})	37 (3.3)	38 (2.8)	0.11	0.92
Ni (μg g^{-1})	3800 (180)	53 (21)	20	< 0.0001
P (%)	0.47 (0.006)	0.55 (0.021)	3.7	0.021
Zn (μg g^{-1})	350 (8.2)	380 (7.8)	2.7	0.054

hyperaccumulating *T. caerulescens* (Brassicaceae) completely deterred feeding by *Pieris brassicae* (Linnaeus) (Lepidoptera: Pieridae) larvae. Partial deterrence by hyperaccumulated Ni was reported by Boyd *et al.* (2002) for snails and Martens & Boyd (1994) for *Pieris rapae* (Linnaeus) (Lepidoptera: Pieridae) larvae. It is unclear how slugs discriminated between high- and low-Ni *S. polygaloides* in our choice test. The lack of visible damage to most high-Ni plants suggests a chemical cue that was detectable to slugs as they crawled over plant surfaces. Unfortunately, high- and low-Ni *S. polygaloides* plants differed in concentrations of several elements other than Ni (Tables 2, 3). Organic constituents also may have varied. For example, Jhee *et al.* (unpublished data) found that *S. polygaloides* grown on high-Ni soil contained as much total glucosinolate as plants grown on low-Ni soil but that the composition of specific glucosinolates varied. Recent tests using snails (*Helix aspersa*) fed the Zn hyperaccumulator *Thlaspi caerulescens* J & C Presl (Brassicaceae) showed that feeding level responded to glucosinolate concentration rather plant Zn concentration (Noret *et al.* 2005). Behmer *et al.* (2005) showed that desert locusts, *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae), develop aversion to artificially flavoured high-Zn food despite being unable to taste Zn. In our experiment, differences in glucosinolate composition of *S. polygaloides* may have been used by slugs to discriminate between hyperaccumulating and non-hyperaccumulating plants.

Although hyperaccumulator levels of Ni have been shown to have defensive effects, the minimum quantity of Ni that can be effective is unclear. Studies of folivorous Lepidoptera using Ni-amended artificial diet have shown toxic thresholds varying from about 1000 μg Ni g^{-1} for *Pieris rapae* (Martens & Boyd 1994) and *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) (Boyd & Moar 1999) to as little as 20 μg Ni g^{-1} for *Plutella xylostella* (Coleman *et al.* 2005). An artificial diet experiment using *Helix aspersa* indicated toxicity at 830 μg Ni g^{-1} but a sublethal effect (reduction in snail mass) was detected at 140 μg Ni g^{-1} (Boyd *et al.* 2002). Few studies have examined the effects of relatively low Ni concentrations (at and below the levels found in accumulator plants) using experiments that feed plants to herbivores. Boyd & Moar (1999) fed leaves of *S. breweri* and *S. tortuosus* to larvae of *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). They showed no toxic effects for leaves from either species grown on high-Ni soils but did find a sublethal effect for high-Ni leaves of *S. tortuosus*, which contained 93 μg Ni g^{-1}. Our experiment with *Limax maximus* found neither toxic (mortality) nor sublethal (mass change) effects of Ni at concentrations < 70 μg Ni g^{-1}. Although these concentrations are above the normal range of Ni in plants, they do not exceed the 100 μg Ni g^{-1} needed to classify them as Ni accumulators. Further experiments using Ni accumulator species are needed to investigate the defensive effectiveness of Ni for plants whose Ni levels are less than those of hyperaccumulators but more than the above-normal levels reported here.

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References

Abacus Concepts (1998) StatView. Cary NC, USA: SAS Institute, Inc

Barker GM (2002) Molluscs as Crop Pests. GB-Wallingford: CAB International

Barker GM (1999) Fauna of New Zealand 38: Naturalized terrestrial Stylommatophora (Mollusca: Gastropoda). Lincoln, Canterbury, New Zealand: Manaaki Whenua Press

Behmer ST, Lloyd CM, Raubenheimer D, Stewart-Clark J, Knight J, Leighton RS, Harper FA, Smith JAC (2005) Metal hyperaccumulation in plants: mechanisms of defence against herbivores. Funct Ecol 19: 55-66

Boyd RS (2004) Ecology of metal hyperaccumulation. New Phytol 162: 563-567

Boyd RS (1998) Hyperaccumulation as a plant defensive strategy. Pp 181–201 *in* Brooks RR (ed) Plants that Hyperaccumulate Heavy Metals. GB-Oxford: CAB International

Boyd RS, Davis MA, Wall MA, Balkwill K (2002) Nickel defends the South African hyperaccumulator *Senecio coronatus* (Asteraceae) against *Helix aspersa* (Mollusca: Pulmonidae). Chemoecology 12: 91–97

Boyd RS, Martens SN (1992) The raison d'être for metal hyperaccumulation by plants. Pp 279–289 *in* Baker AJM, Proctor J, Reeves RD (eds) The Vegetation of Ultramafic (Serpentine) Soils. GB-Andover, Hants: Intercept

Boyd RS, Martens SN (1994) Nickel hyperaccumulated by *Thlaspi montanum* var. *montanum* is acutely toxic to an insect herbivore. Oikos 70: 21–25

Boyd RS, Martens SN (1999) Aphids are unaffected by the elemental defense of the nickel hyperaccumulator *Streptanthus polygaloides* (Brassicaceae). Chemoecology 9: 1–7

Boyd RS, Moar WJ (1999) The defensive function of Ni in plants: response of the polyphagous herbivore *Spodoptera exigua* (Lepidoptera: Noctuidae) to hyperaccumulator and accumulator species of *Streptanthus* (Brassicaceae). Oecologia 118: 218–224

Bruelheide H, Scheidel U (1999) Slug herbivory as a limiting factor for the geographical range of *Arnica montana*. J Ecol 87: 839–848

Coleman CM, Boyd RS, Eubanks MD (2005) Extending the elemental defense hypothesis: dietary metal concentrations below hyperaccumulator levels could harm herbivores. J Chem Ecol (in press)

Frank T (2003) Influence of slug herbivory on the vegetation development in an experimental wildflower strip. Basic Appl Ecol 4: 139–147

Grewal SK, Grewal PS, Hammond RB (2003) Susceptibility of North American native and non-native slugs (Mollusca: Gastropoda) to *Phasmarhabditis hermaphrodita* (Nematoda: Rhabditidae). Biocontrol Sci Technol 13: 119–125

Hickman JC (ed) (1993) The Jepson Manual: Higher Plants of California. Berkeley, California, USA: University of California Press

Huitson SB, Mcnair MR (2003) Does zinc protect the zinc hyperaccumulator *Arabidopsis halleri* from herbivory by snails? New Phytol 159: 453–459

Jhee EM (2004) Hyperaccumulation of nickel by *Streptanthus polygaloides* (Brassicaceae): implications for elemental defense. PhD dissert, Auburn Univ, Alabama, USA

Kruckeberg AR (1984) California Serpentines: Flora, Vegetation, Geology, Soils and Management Problems. Berkeley, California, USA: University of California Press

Kruckeberg AR, Reeves RD (1995) Nickel accumulation by serpentine species of *Streptanthus* (Brassicaceae): field and greenhouse studies. Madroño 42: 458–469

Martens SN, Boyd RS (2002) The defensive role of Ni hyperaccumulation by plants: a field experiment. Am J Bot 89: 998–1003

Martens SN, Boyd RS (1994) The ecological significance of nickel hyperaccumulation: a plant chemical defense. Oecologia 98: 379–384

Noret N, Meerts P, Tolrà R, Poschenrieder C, Barceló J, Escarre J (2005) Palatability of *Thlaspi caerulescens* for snails: influence of zinc and glucosinolates. New Phytol 165: 763–772

Pollard AJ, Baker AJM (1997) Deterrence of herbivory by zinc hyperaccumulation in *Thlaspi caerulescens* (Brassicaceae). New Phytol 135: 655–658

Reeves RD (1992) The hyperaccumulation of nickel by serpentine plants. Pp 253–277 *in* Baker AJM, Proctor J, Reeves RD (eds) The Vegetation of Ultramafic (Serpentine) Soils. GB-Andover, Hants: Intercept

Reeves RD, Baker AJM. 2000. Metal-accumulating plants. Pp 193–229 *in* Raskin I, Ensley BD (eds) Phytoremediation of Toxic Metals. New York, New York, USA: John Wiley

Reeves RD, Brooks RR, Macfarlane RM (1981) Nickel uptake by Californian *Streptanthus* and *Caulanthus* with particular reference to the hyperaccumulator *S. polygaloides* Gray (Brassicaceae). Am J Bot 68: 708–712

Rodriguez MA, Brown VK (1998) Plant competition and slug herbivory: Effects on the yield and biomass allocation pattern of *Poa annua* L. Acta Oecol 19: 37–46

Shapiro AM (1981) Egg-mimics of *Streptanthus* (Cruciferae) deter oviposition by *Pieris sisymbrii* (Lepidoptera: Pieridae). Oecologia 48: 142–143

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