ECOLOGICAL AND EVOLUTIONARY CONSEQUENCES OF PLANT GROWTH ON SERPENTINE SOIL: EFFECTS OF SOIL METALS ON PLANT MORPHOLOGY, METAL ACCUMULATION, PLANT-POLLINATOR INTERACTIONS, AND POLLEN-PISTIL INTERACTIONS

by

George A. Meindl

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MA, Humboldt State University, 2009

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This dissertation was presented

by

George A. Meindl

It was defended on

November 3, 2014

and approved by

Dr. Daniel Bain, Dept. of Geology and Planetary Science, University of Pittsburgh

Dr. Walter Carson, Dept. of Biological Sciences, University of Pittsburgh

Dr. Susan Kalisz, Dept. of Biological Sciences, University of Pittsburgh

Dr. Rick Relyea, Dept. of Biological Sciences, University of Pittsburgh

Dissertation Advisor: Dr. Tia-Lynn Ashman, Dept. of Biological Sciences, University of Pittsburgh

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George A. Meindl, PhD

University of Pittsburgh, 2014

Edaphic factors are a strong selective force in shaping both plant species distributions and the diversification of many lineages. Specifically, adaptation to novel soil environments can result in species-level changes in floral morphology, phenology, or chemistry, each of which may affect plant reproduction. However, whether floral chemical changes alter plant reproduction following colonization of novel soils is poorly described. In this work, I investigate the effects of soil chemistry on plant chemistry, plant-animal interactions, and pollen-pistil interactions using serpentine-adapted plant species to help determine the effects of the soil chemical environment on plant reproduction and reproductive isolation. I show that (1) plants accumulate soil metals into vegetative and reproductive organs, as well as into pollen and nectar, (2) floral metal accumulation deters generalist pollinators and filters natural pollinator communities, and (3) floral metal accumulation alters pollen grain germination. These findings have important implications for plant reproduction on metal-rich soils. For example, my research has identified two novel mechanisms through which serpentine soil chemistry may foster reproductive isolation between species or populations growing in disparate soil environments. First, floral metal accumulation may result in pollinator filtering. Specifically, closely related plant species occurring in sympatry that differ in floral metal accumulation may become reproductively isolated through reduced pollinator sharing. Second, floral metal accumulation may provide a

mechanism through which gene flow is reduced between serpentine and non-serpentine populations by altering pollen germination and pollen-pistil compatibility. I found that elevated metal concentrations in the pistils of maternal plants limits pollen tube growth towards ovules in non-adapted species. Furthermore, my results suggest that using metal hyperaccumulating plants in phytoremediation should be considered with caution. While I found that generalist pollinators exhibited decreased visitation to Ni-enriched flowers, they still visited these flowers, and therefore likely ingested a potentially toxic resource. If bioaccumulation of heavy metals occurs in plant-pollinator systems near metal-contaminated soils, pollinator populations may become threatened. This study highlights the influence of the soil environment on plant ecological interactions and plant evolution, and elucidates the role of the edaphic factor on plant reproduction.

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PREFACE

This dissertation is dedicated to all of my family and friends who have supported me from the beginning. As educators themselves, my parents Robert and Marcia have motivated me to pursue higher education, and without their love, support, and guidance I would not have made it this far. Jess Hua inspires me to better myself every day, and I thank her for her unyielding love and support. None of this work would have been possible without the guidance of my committee members, and, especially, my adviser Dr. Tia-Lynn Ashman. Tia-Lynn is the epitome of a scholar, and I am grateful to have found my way to her lab. Finally, I would like to thank the following people for various contributions: P. Aigner, C. Koehler and R. Woerly of the McLaughlin Natural Reserve, J. Wenzel of the Powdermill Nature Reserve, and G. Arceo-Goméz, M. Koski and C. Alonso for providing assistance at field sites. K. DeHart, R. McGown, C. Murray, and H. Wipf assisted with plant tissue element analysis. N. Boehme, J. Pitts, J. Wenzel and J. Rawlins assisted with insect identification. E. York, K. Schuller, and K. Mazzaferro assisted with greenhouse studies. Both former and current members of the Ashman lab, especially G. Arceo-Goméz, M. Koski, R. Spigler, N. Forrester, R. Govindarajulu, and K. LeCroy, contributed much to this thesis.

1.0 INTRODUCTION

Understanding how abiotic factors influence species abundance, distribution and diversification are central questions in both ecology and evolution (Schluter 2009). For plants, edaphic factors are thought to be a strong selective force in shaping both species distributions and the diversification of many lineages (Rajakaruna 2004). Specifically, adaptation to novel soil environments can lead to species-level changes in floral morphology, phenology, or chemistry, each of which may affect plant reproduction and population growth or persistence (Brady et al. 2005). While the reproductive consequences of altered floral morphology and phenology following colonization of novel soils have been studied (Brady et al. 2005; Bomblies 2010), whether floral chemical changes alter plant reproduction is less well understood. However, effects of soil chemistry on plant reproduction may be profound. For example, differences in floral chemistry between closely related species may lead to pollinator sorting (Adler 2000) and ultimately reproductive isolation. Differences in floral chemistry between species or populations may also limit hybridization as the pollen and ovules of species may vary in their tolerance to certain elements found in excess in soils and, consequently, in plant reproductive tissues (e.g., heavy metals; Searcy and Mulcahy 1985). Thus, soil chemistry can directly alter the evolutionary trajectory of species through modifying plant-pollinator or pollen-pistil interactions.

Heavy metals occur naturally in trace concentrations in soils, but some soil environments, including both natural and anthropogenically altered, contain elevated concentrations that are toxic to many organisms (Nagajyoti et al. 2010). For example, serpentine-derived soils represent a nutritionally stressful growing environment for most plants because of a low Ca:Mg ratio, deficiency of essential nutrients (e.g., N, P, K), and high levels of potentially phytotoxic heavy metals (e.g., Ni, Co, Cr; Brooks 1987; Brady et al. 2005; Kazakou et al. 2008). The elevated heavy metal concentrations, in particular, in serpentine soils are thought to drive levels of plant adaptation (Lazarus et al. 2011), as non-adapted species lack physiological mechanisms to avoid metal toxicity (Kazakou et al. 2008). While some plants require Ni in trace quantities as an active component of the enzyme urease (Welch 1981), it is generally considered toxic to plants and is implicated in causing abnormal vegetative growth, necrosis and chlorosis of leaves and inhibiting photosynthesis when present in excess in soils (reviewed in Yusuf et al. 2011). As a result, most serpentine-adapted plant species exclude metals (DeHart et al. 2014), but some are known to accumulate metals into above ground tissues at high concentrations (i.e., metal hyperaccumulators; van der Ent et al. 2013). Metal accumulation and hyperaccumulation has been hypothesized to serve several fitness-related functions in plants, including allelopathic effects and defense against herbivores (Boyd 2007). However, the direct reproductive consequences of metal accumulation, particularly floral metal accumulation, are unclear.

There are two primary reasons for studying floral metal accumulation. First, phytoremediation is a green technology that uses metal hyperaccumulating plants to clean up metal-contaminated soils. Soil metal pollution not only negatively impacts plants, but also microorganisms (Acikel and Alp. 2010; Azarbad et al. 2013), fungi (Leyval et al. 1997), insects and other animals (Cohn et al. 1992; Timmerman et al. 1992; Hanson et al. 2004;

Warchalowska-Silwa et al. 2005; Sorvari et al. 2007; Van Ooik et al. 2007), including humans (e.g., metal ingestion can cause cancer, via formation of free radicals in cells; Tchounwou et al. 2012). Thus, identifying cost-effective and environmentally friendly means to remediate polluted soils is a research priority (Chaney et al. 2010). However, considering metals are toxic to insects, and some pollinating insects, including honey bees, are in decline (Potts et al. 2010), it is important to assess the ecological risks of phytoremediation before implementing it on a large scale. Second, changes in floral chemistry following adaptation to novel soil environments may impact patterns of pollen transfer within natural plant populations, either by altering plant-pollinator interactions or pollen-pistil interactions. Serpentine soils, which can be found on every continent (Brooks 1987), provide one of the most remarkable examples of plant adaptation to atypical soils (O'Dell and Rajakaruna 2011) and geographic regions containing serpentine often harbor numerous endemic species (Brooks 1987; Safford et al. 2005; Anacker 2011). Plant response to the heavy metal Ni found in serpentines is relatively well studied (Kazakou et al. 2008), and provides an excellent model to test whether heavy metal accumulation adversely affects plant reproduction. Documenting the effects of floral chemistry on plant reproduction may help to explain plant species distribution, abundance and diversification on both natural (e.g., serpentine) and anthropogenically induced metal-rich soils.

In this work, I investigate the effects of soil chemistry on plant morphology, chemistry, plant-animal interactions, and pollen-pistil interactions using serpentine-adapted plant species to help elucidate effects of the soil chemical environment on plant reproduction. First, I determine the effects of serpentine soil chemistry on plant morphology, chemistry, and plant-animal interactions for a serpentine-tolerant plant species (*Mimulus guttatus*; chapter I). Second, I determine the degree to which several plant species from the mustard family (Brassicaceae) that

vary in affinity to serpentine soil accumulate Ni into leaves, flowers, and pollinator rewards (chapters II, III). Next, I evaluate whether floral Ni accumulation alters plant-pollinator interactions in both natural and experimental settings, focusing on the Ni hyperaccumulating species *Streptanthus polygaloides* (chapters IV, V, VI). Finally, I determine the effects of floral metal accumulation on plant reproduction (i.e., pollen germination, fruit and seed production) for two species known to differ in magnitude of floral metal accumulation (chapter VII).

Notably, results from this work show that (1) plants accumulate soil metals into both vegetative and reproductive organs, as well as into pollen and nectar, (2) floral metal accumulation deters generalist pollinators and filters natural pollinator communities associated with metal hyperaccumulating plants, and (3) floral metal accumulation alters pollen grain germination, particularly for species that are not endemic to metal-rich soils. These findings have important implications for plant reproduction on naturally metal-rich soils, including serpentines, as well as soils polluted by human activity. For example, my results suggest that using metal hyperaccumulating plants for the purpose of phytoremediation should be considered with caution. Despite the apparent benefits of phytoremediation, land managers have not fully considered how interactions with pollinators may be affected. While I found that generalist pollinators visited fewer flowers and spent less time foraging on flowers with Ni-enriched nectar and pollen, they still visited metal-rich flowers, and therefore likely ingested a potentially toxic resource. If bioaccumulation of heavy metals occurs in plant-pollinator systems near metal-contaminated soils, pollinator populations may become threatened in these areas. Understanding the ultimate fate of soil metals is therefore critical not only for plants, but also for the animals that use them as resources.

For plants growing in naturally metal-rich soils, my research has identified two novel mechanisms through which soil chemistry may foster reproductive isolation between species or populations growing in disparate soil environments. First, I provide novel evidence that metal hyperaccumulating plants host unique species of pollinating insects, relative to closely related plants occurring in sympatry. Floral metal accumulation may result in pollinator filtering, thus closely related plant species occurring in sympatry that differ in floral metal accumulation may become reproductively isolated. Previous studies have found correlations between edaphic shifts and pollination system shifts for plant sister species with overlapping geographic ranges (Niet et al. 2006)- my work highlights one possible mechanism by which these shifts may occur, i.e., differences in floral chemistry following adaptation to novel soils leads to reduced pollinator sharing. Second, results from this study suggest that floral metal accumulation may provide a mechanism through which gene flow is reduced between serpentine and non-serpentine populations by altering pollen-pistil compatibility. Specifically, I found that non-endemic serpentine plants grown in high-Ni soils displayed decreased pollen germination, fruit and seed production relative to plants grown in low-Ni soils. This suggests that pollen arriving from a non-serpentine plant is unlikely to be successful in siring progeny, as the Ni concentrations in the pistils of maternal plants may limit pollen grain germination and/or pollen tube growth towards ovules. While soil heavy metals have been previously implicated in fostering reproductive isolation between populations on vs. off metal-rich soil indirectly via changes in floral phenology (Antonovics 2006), here I provide evidence for a more direct mechanism through which soil metals may impart reproductive isolation between populations. Similar to what has been observed for the metal Cu in *Mimulus guttatus* (Searcy and Mulcahy 1985; Searcy and Macnair 1990), floral Ni accumulation may provide a selective barrier to gene exchange between

serpentine and non-serpentine populations, and thus provide a prezygotic isolating mechanism between populations that vary in floral metal concentrations and/or metal tolerance. Edaphic islands, such as serpentine soils, provide a model setting to determine the influence of the abiotic environment on plant ecological interactions and plant evolution, and continued study of the effects of soil chemistry on plant reproduction will help elucidate the role of the edaphic factor on plant adaptation and speciation.

2.0 EDAPHIC FACTORS AND PLANT-INSECT INTERACTIONS: DIRECT AND INDIRECT EFFECTS OF SERPENTINE SOIL ON FLORIVORES AND POLLINATORS

2.1 INTRODUCTION

Biotic interactions can be influenced by abiotic factors, thus identical communities found in disparate environments (i.e., with different resource availability) may differ in both the strength (Breitburg et al. 1997; Alonso 1999; Chalcraft and Andrews 1999) and direction (Pugnaire and Luque 2001) of interactions. Abiotic conditions have been documented to alter biotic interactions across a broad range of organisms, including effects of temperature and moisture on insect (Park 1954) and bivalve competition (Connell 1961). Plants, in particular, are heavily dependent upon their abiotic environment for inorganic nutrient acquisition, and as a consequence may be particularly susceptible to abiotic-mediated variation (Klanderud and Totland 2005) in morphology and plant tissue chemistry, which in turn may affect how they interact with animals. Chemical and physical aspects of soils are extremely variable and this variation can alter both plant morphology and tissue chemistry (e.g., Cunningham et al. 1999; Murren et al. 2006; Burnett et al. 2008). For plants that occur in a variety of soil types, it is unclear whether interactions with mutualists (e.g., pollinators) or antagonists (e.g., herbivores) are affected by soil context, and whether soil could modify these interactions via direct effects on

plant chemistry or indirect effects on morphology. However, such modification of biotic interactions could be instrumental in varying patterns of coevolution (i.e., the geographic mosaic of coevolution; Thomson 1999).

The soil environment can influence plant reproductive morphology, which can in turn affect both plant-florivore and plant-pollinator interactions. Macronutrients in the soil, such as N, P, K, Ca and Mg, have been shown to influence flower size and number (e.g., Nagy and Proctor 1997; Murren et al. 2006; Burnett et al. 2008). In addition, toxic elements, such as heavy metals, often result in stunting of growth when present in high concentrations in the soil (Antonovics et al. 1971) and are also known to influence flower size (Hladun et al. 2011) and flower number (Saikkonen et al. 1998). Soil-induced changes in floral morphology can have consequences for plant reproduction, as both pollinators (Mitchell et al. 2004; Ivey and Carr 2005) and herbivores (Juenger et al. 2005; Ashman and Penet 2007) generally favor plants with large floral displays. As a result, soil chemistry may mediate the quantity and quality of plant interactions with florivores and pollinators. Many studies have documented the effects of environment on plant reproductive morphology, yet few (e.g., Galen 2000; Lau et al. 2008) have determined whether these morphological changes lead to altered plant-animal interactions across different environments.

Soil chemistry also can have effects on plant tissue chemistry (Cunningham et al. 1999), although the consequences for plant-animal interactions are less well understood. While plant-herbivore interactions are often studied in the context of plant secondary compounds (reviewed in Mithöfer and Boland 2012), recent studies suggest that primary metabolites (e.g., N, P, K) may also greatly influence both herbivore preference (Alonso and Herrera 2003) and fitness (Beanland et al. 2003; Perkins et al. 2004). In addition, soils that contain toxic elements can alter

plant-herbivore interactions. For instance, *Streptanthus polygaloides*, a serpentine soil endemic, hyperaccumulates Ni (i.e., tissues >1,000 ppm Ni; Baker and Brooks 1989), which results in less leaf damage by herbivores (Boyd and Moar 1999) and pathogens (Boyd et al. 1994). The effect of metal accumulation on plant-pollinator interactions, however, is unclear. For example, interactions with pollinators may also be affected if metals are translocated to floral tissues and pollinator rewards (e.g., nectar and pollen). A recent study of a non-metal (Se) hyperaccumulator has shown that flower constituents, including nectar, can accumulate non-essential elements in high concentrations (Hladun et al. 2011), though metal accumulators from serpentines have not been similarly studied, and the implications of floral metal and metalloid accumulation on plant-pollinator interactions have only begun to be explored (Quinn et al. 2011). Moreover, serpentine soils may generally influence plant-animal interactions (i.e., for non-hyperaccumulating plants) through changes in tissue chemistry, as concentrations of metals in plant tissues far below hyperaccumulator thresholds have been shown to be toxic to herbivores (Coleman et al. 2005). In addition, while studies of folivory are important, an understanding of how flower chemistry alters florivory is needed, as many insects supplement their diets with nutrient-rich flower tissue (Held and Potter 2004). In fact, some studies suggest that florivory can be just as common as leaf herbivory in natural populations (e.g., Zangerl and Rutledge 1996; Wolfe 2002), with potentially negative implications for plant reproductive success (Mothershead and Marquis 2000). Florivores can affect male and female fitness both directly, through consumption of gamete-housing structures, and indirectly by altering floral traits important for biotic interactions (e.g., pollinator attraction; reviewed in McCall and Irwin 2006). Therefore, understanding how the soil environment alters plant-florivore interactions may be vital towards explaining plant adaptation to unique soils.

Serpentine soil is distinct from adjacent soils by having low Ca:Mg ratios, mineral nutrient deficiencies (e.g., P, K) and relatively high concentrations of several metals (Co, Cr, Ni, Fe, Mg, and Zn; Brady et al. 2005; Safford et al. 2005; Table S1). These soils provide an ideal model system to test whether the soil environment alters plant-florivore and plant-pollinator interactions as they are (i) globally distributed, (ii) host many species of tolerant plants and (iii) are chemically distinct from adjacent soil types (Brady et al. 2005; Harrison and Rajakaruna 2011). We address whether biotic interactions are soil-dependent by answering the following questions with respect to a serpentine tolerant species, *Mimulus guttatus*: (1) Does growth on serpentine soil alter traits that mediate plant-animal interactions, i.e., flower size, flower number, and inflorescence height? (2) Does serpentine soil directly influence floral chemistry, specifically for minerals known to be enriched or deficient in serpentine and/or known to influence plant-animal interactions (macronutrients: Ca, Mg, P, K; micronutrients: Fe, Ni, Zn, B; or other beneficial nutrients: Al, Na; Marschner 1986)? (3) Is (a) pollinator visitation rate, (b) pollinator diversity, and/or (c) florivore damage lower for plants growing in serpentine vs. non-serpentine soils?

2.2 METHODS

2.2.1 Study System

Mimulus guttatus (Phrymaceae) is a widespread herbaceous plant native to western North America that inhabits creeks or seepage areas (Vickery 1978). It can grow on serpentine and

non-serpentine soil (Vickery 1978), and thus is regarded as a serpentine tolerant species (Gardner and Macnair 2000). It is self-compatible and predominantly pollinated by bees, including honeybees (*Apis mellifera*) and bumblebees (*Bombus* spp.), although it is also visited by beetles, flies and butterflies (Gardener and Macnair 2000; Meindl, Arceo-Gomez, and Ashman, unpublished data). Its flowers are damaged by insect florivores including grasshoppers (Orthoptera) and beetles (e.g., Buprestidae and Melyridae; G. A. Meindl, pers. obs.).

2.2.2 Study Sites

This study was conducted in serpentine ('S') and non-serpentine ('NS') seeps at the Donald and Sylvia McLaughlin Natural Reserve in Napa and Lake counties of California. We studied *M. guttatus* in two serpentine (S1: 38°51' N 122°25' W and S2: 38°51' N 122°27' W) and two non-serpentine seeps (NS1: 38°51' N 122°22' W and NS2: 38°52' N 122°26' W), separated by one to five km, in May-August of 2010 and 2011. Soils at the two types of seeps are chemically distinct in most major macro- and micronutrients (Table 1; http://nrs.ucdavis.edu/McL/natural/geology/index.html). Specifically, serpentine soil at the study sites was higher in Fe, Mg, Ni and Zn (56%, 326%, 422% and 69% respectively), lower in Al, Ca, K and P (27%, 44%, 39% and 49% respectively) than non-serpentine soil, but similar in B and Na (Table 1). While bioavailable fractions of Ni in serpentine soils at McLaughlin are lower compared to other serpentine sites (e.g., Oze et al. 2008), concentrations of Ni are higher in serpentine soils relative to non-serpentine soils on the reserve (Wright et al. 2006; Table 1).

Each seep supported several hundred *M. guttatus* along with *Vicia villosa, Melilotus alba* (Fabaceae), *Torilis arvensis* (Apiaceae), and *Stachys ajugoides* (Lamiaceae) at non-serpentine

seeps and *Castilleja rubicundula* (Scrophulariaceae), *Triteleia peduncularis* (Liliaceae), *Lotus micranthus* (Fabaceae), and *Plagiobothrys stipitatus* (Boraginaceae) at serpentine seeps. *Zigadenus venenosus* (Liliaceae) and *Trifolium obtusiflorum* (Fabaceae) were present at both seep types.

2.2.3 Abiotic and Biotic Interactions in Natural Populations

2.2.3.1 Floral display/flower chemistry

We assessed whether plants on serpentine and non-serpentine soil differed in aspects of floral display. We established four to six 1x2 m plots at five m intervals along each of seven transects/seep over the course of two seasons. At mid-bloom, we measured corolla width (widest distance across lower lip of corolla to the nearest 0.1 mm; Robertson et al. 1994) and inflorescence height (mm) with digital calipers, number of open flowers per inflorescence and the percent of open flowers with visible florivore damage (i.e., corolla tissue missing) on three plants in a standard position in each plot. Corolla width was always measured on the second most recently opened flower on an inflorescence. Trait averages for corolla width, inflorescence height and the number of open flowers per inflorescence were calculated for each of 164 plots (42 at NS1 and S2; 40 at NS2 and S1).

To determine whether flower tissue chemistry differed between serpentine and non-serpentine plants, in 2010 we bulk-collected entire, freshly opened flowers during peak flowering from 10 plots/site. These were rinsed with diH_2O and dried at 60°C for 48 hours. A 0.1 g sample (4-5 flowers) was microwave digested in 4 mL of trace metal grade HNO_3 and brought to a final volume of 15 mL with MilliQ (Millipore, Bedford, MA, USA) H_2O (Esslemont et al.

2000). We analyzed element composition using inductively coupled plasma mass spectrometry (ICP-MS, Perkin/Elmer NEXION 300X), and present data on 10 elements known to differ between serpentine and non-serpentine soils and/or known to influence plant-animal interactions (macronutrients: Ca, Mg, P, K, micronutrients: Fe, Ni, Zn, B; other beneficial: Al, Na; e.g., Alonso and Herrera 2003; Beanland et al. 2003; Wang and Mopper 2008; Pilon-Smits et al. 2009).

We analyzed corolla width, inflorescence height, number of open flowers per inflorescence and floral tissue chemistry with mixed linear models (PROC MIXED; SAS 2010) with soil type (serpentine vs. non-serpentine) and year (2010, 2011) as factors. Soil type and year (for display traits only) were fixed effects, while site identity (nested within soil type) was a random effect. To control for potential Type I errors due to multiple comparisons, we used Bonferroni corrections to adjust alpha levels. The data for elements B, Na, Al, Fe, and Zn were log transformed to improve normality.

2.2.3.2 Pollinators

To determine whether pollinator visitation rate differed between serpentine and non-serpentine *M. guttatus*, 10-10.5 hrs of observation/site was conducted across three (2010) to four (2011) days/site in June/July. Observations were conducted for 15 min/plot between the hours of 10 a.m. and 4 p.m. on sunny days. For each week of observation, the order that serpentine and non-serpentine sites were observed was reversed, and sites were visited alternately in the morning and afternoon on successive days of observation. For each plot, we recorded the number of *M. guttatus* flowers and two 'context' characters: the number of heterospecific flowers and percent bare ground (a measure of plant density), for use as covariates as these might also influence

visitation (e.g., Bernhardt et al. 2008; Duffy and Stout 2011). Visitors to *M. guttatus* flowers were recorded as small bees, medium bees, large bees (including honeybees and bumblebees) and beetles. Unknown visitors were collected and later identified to species or family. We calculated visitation rate as the number of visits/flower/hour, pooled across all visitors. To distinguish flower visitors foraging for nectar and pollen rewards from florivores we refer to them as 'pollinators', but acknowledge that some of these flower-visiting insects may not be effective pollinators.

The effect of soil type on visitation rate to *M. guttatus* was determined using mixed linear models (PROC MIXED; SAS 2010), with site soil type (serpentine vs. non-serpentine) and year (2010, 2011) as fixed factors, and site identity (nested within site soil type) as a random effect. Average *M. guttatus* floral display (i.e., corolla width, number of open flowers per inflorescence, and inflorescence height) per plot, time of day of observations, and context characters (i.e., the number of heterospecific flowers and percent bare ground) were included as covariates. Visitation rate was transformed (log (visitation rate + 1)) to meet the assumption of normality. Composition of the pollinator assembly, pooled across all observations/year, to *M. guttatus* growing on serpentine and non-serpentine was compared using two-way chi-square analysis (PROC FREQ; SAS 2010) with visitor type and soil type as factors.

2.2.3.3 Florivores

To assess whether *M. guttatus* on serpentine and non-serpentine soil differed in terms of florivore damage, we measured the percent of open flowers with corolla tissue missing on three plants/plot. Average florivore damage/plot was analyzed using a generalized linear mixed model (PROC GLIMMIX; SAS 2010) with site soil type and year as factors and display traits (corolla

width, number of open flowers per inflorescence and inflorescence height) as covariates. Site soil type and year were fixed effects, while site identity (nested within site soil type) was a random effect.

2.2.4 Abiotic and Biotic Interactions for Experimental Plants

2.2.4.1 Floral display/flower chemistry

To isolate the specific effects of soil on reproductive morphology and/or chemistry, we conducted a common garden reciprocal soil transplant experiment using field-collected seedlings and soil. Soil from the two serpentine seeps was mixed together in equal proportions to create a generic serpentine soil, and soil from the two non-serpentine sites was similarly treated to create the non-serpentine soil. All soils were augmented with 15% vermiculite (Perlite Vermiculite Packaging Industries Inc., OH, USA) to increase water-holding capacity in 27 cm^3 'rocket' pots (Deepots, Stuewe and Sons, Inc.). Fifty *M. guttatus* seedlings, in the two-cotyledon stage, were collected from each seep and assigned randomly to one of the soil treatments. These were arranged in 25 blocks of eight plants (one each per site-treatment combination; total N=200) on an outside bench and bottom watered as needed. Field-collected seedlings were used in these experiments so they would be phenologically synchronized with the natural populations. We measured corolla width (mm) for the first three flowers produced by each plant, and inflorescence height (mm) and number of open flowers per plant two weeks after the first flower opened. The first 15 freshly opened flowers per plant were bulk-collected for elemental analysis as above.

To determine whether soil type affected corolla width, number of open flowers per inflorescence, or inflorescence height, we used mixed linear models (PROC MIXED; SAS 2010) with experimental soil type, source population soil type, and their interaction as factors. Experimental soil type and source population soil type were treated as fixed effects, while source population (nested within source population soil type) and block were treated as random effects. We used separate mixed linear models (PROC MIXED; SAS 2010) for each element in floral tissue as above. To control for potential Type I errors due to multiple comparisons, we used Bonferroni corrections to adjust alpha levels. The data for elements B, Al, Fe, Ni, and Zn were log transformed to improve normality.

2.2.4.2 Pollinators

To determine whether pollinator preference depends on soil type, we created arrays of inflorescences. Inflorescences were collected from both serpentine and non-serpentine sites, corolla width and flower number recorded, and placed in 225 mL centrifuge tubes filled with water and topped with florist's foam. Each array consisted of two inflorescences from each site evenly spaced in a circle with a circumference of 52 cm. On each day of observation, two arrays were placed within the four sites and observed for six fifteen-minute intervals (18 hrs of total; N=6 arrays per site; total N=24 arrays). Following each observation interval, the position of the arrays was switched. Visitation to each inflorescence was recorded as visits/flower/hour.

The effect of site soil type on pollinator visitation rate to inflorescences of *M. guttatus* within arrays was determined using a mixed linear model (PROC MIXED; SAS 2010). The model included site soil type and source population soil type as factors and corolla width and time of day as covariates. Site soil type and source population soil type were treated as fixed

effects, while site identity (nested within site soil type) and source population (nested within source population soil type) were treated as random effects. Visitation rate to inflorescences was transformed (log (visitation rate + 1)) to meet the assumption of normality.

2.2.4.3 Florivores

We placed arrays of 16-20 potted plants, half grown in serpentine and half grown in non-serpentine soil, within each site for 72 hrs. After exposure, florivore damage was estimated as percent of corolla removed on a 0-5 scale (where 0=no damage, 1 up to 20%, 2=20-40%, 3=40-60%, 4=60-80% and 5=80-100%). One array/site was set out for three consecutive weeks (208 total plants).

The proportion of plants placed within serpentine vs. non-serpentine sites that received damage from florivores was compared using a two-way chi-square analysis (PROC FREQ; SAS 2010) with site soil type and florivore damage (present or absent) as factors. For those *M. guttatus* plants that received damage by florivores, florivore damage score was analyzed using a generalized linear mixed model (PROC GLIMMIX; SAS 2010). The model included site soil type and source population soil type as factors and corolla width and inflorescence height as covariates. Site soil type and source population soil type were treated as fixed effects, while site identity (nested within site soil type) and source population (nested within source population soil type) were treated as random effects.

2.3 RESULTS

2.3.1 Abiotic and Biotic Interactions in Natural Populations

2.3.1.1 Floral display/flower chemistry

Serpentine soil influenced floral display in *M. guttatus.* Plants growing on serpentine soils produced 60% shorter inflorescences, 12% smaller corollas and 52% fewer open flowers per inflorescence (Table 1; Fig. 1) than those growing on non-serpentine. Differences between years were also evident for mean corolla width (2010: 22.08 ± 0.2 mm; 2011: 20.92 ± 0.23 mm) and mean number of open flowers per inflorescence (2010: 4.28 ± 0.2; 2011: 3.87 ± 0.18; Table 2).

Flowers of *M. guttatus* on serpentine soil differed in chemical content from those on non-serpentine soil. Floral tissue was more concentrated in Mg (29%), but less concentrated in Ca, P and K (39%, 43%, and 22% respectively) than those of non-serpentine plants (Fig. 2; Table 3). Flowers on serpentine plants were more concentrated in Zn (42%) and Na (97%), but less concentrated in Fe, Ni, Al and B (16%, 15%, 33%, and 42% respectively) than those produced by non-serpentine plants (Fig. 2; Table 3).

2.3.1.2 Pollinators

Visitation rates differed between serpentine and non-serpentine *M. guttatus*. Plants growing on non-serpentine received three times more pollinator visits per flower per hour by all insects pooled relative to plants growing in serpentine populations (Fig. 3; Table 4), and this difference exists even after corolla width was accounted for (Table 4). There was a difference in visit rate between years (2010 vs. 2011: 0.63 ± 0.08 vs. 0.73 ± 0.07; Table 4).

Pollinator assemblage differed between serpentine and non-serpentine seeps in both 2010 (χ^2=51.47, df=3, P<0.0001) and 2011 (χ^2=29.68, df=3, P<0.0001). Across both years, large bees and beetles made up a larger percentage of all pollinators observed on *M. guttatus* at non-serpentine seeps than at serpentine seeps (large bees: 26% vs. 10%; beetles: 18% vs. 3%; Tables 5, 6).

2.3.1.3 Florivores

Flowers of serpentine plants received 60% less damage than non-serpentine plants, though this difference was only marginally statistically significant ($F_{1,2}$=6.32; P=0.064; Fig. 3; Table 7). Similar to pollinator visitation, floral display also influenced florivore damage in natural populations, as flower number and inflorescence height explained a significant amount of the variation in damage amount (Table 7).

2.3.2 Abiotic and Biotic Interactions for Experimental Plants

2.3.2.1 Floral display/flower chemistry

Similar to the natural populations, serpentine soil affected floral display traits of *M. guttatus* in the transplant experiment, and this was true regardless of their soil-type origin (experimental soil type x source population soil type interaction: all $P > 0.1$). Plants grown in serpentine soil produced 12% shorter inflorescences and 22% smaller corollas relative to plants grown in non-serpentine soil, but there was no difference in open flowers per inflorescence (Fig. 4; Table 8).

In addition, flowers from *M. guttatus* growing in serpentine soil were found to be chemically distinct from those on non-serpentine soil, regardless of the soil type in which they

originated. Floral tissues of plants on serpentine were more concentrated in Mg (34%), but less concentrated in Ca, P and K (39%, 24% and 18% respectively) compared to those grown on non-serpentine soil (Fig. 2; Table 9). For micronutrients and other beneficial elements, floral tissues of plants on serpentine were more concentrated in Zn (8%) and Na (24%), but less concentrated in Fe, Al, Ni and B (36%, 33%, 19% and 19% respectively) compared to those on non-serpentine soil (Fig. 2; Table 9). In one case, there was a significant experimental soil type by source population soil type interaction, where plants from non-serpentine populations accumulated more Fe into flowers when grown on non-serpentine soil compared to all other plants (Table 9).

2.3.2.2 Pollinators

Regardless of where inflorescences were collected from, insects visited arrays placed at non-serpentine sites three times more often than those placed at serpentine sites (Table 10). Source population soil type did not influence pollinator visitation rates to flowers in arrays (Table 10). Pollinator visitation increased with flower size regardless of source population soil type (Table 10).

2.3.2.3 Florivores

There was a strong effect of site soil type on the frequency of florivore damage: 19% of plants placed at serpentine sites received florivore damage, compared to 40% at non-serpentine sites (χ^2=8.83, df=1, P<0.01). Moreover, flowers of potted plants growing in serpentine soil received 34% less damage compared to plants growing in non-serpentine soil, a marginally significant difference ($F_{1,2}$=16.59; P=0.055; Fig. 5; Table 11). Neither flower size nor inflorescence height, however, influenced the amount of damage by florivores (Table 11).

2.4 DISCUSSION

Our study simultaneously shows that serpentine soil alters plant-insect interactions both directly, through plant tissue chemistry, and indirectly, through floral display, and thus it adds a new dimension to the growing body of work on the effect of serpentine on plant morphology and chemistry (Harrison and Rajakaruna 2011). While plant-mycorrhizae interactions across serpentine and non-serpentine plant populations are beginning to receive attention (Schechter and Bruns 2008; Davoodian et al. 2012), plant-pollinator and plant-florivore interactions across populations of serpentine tolerant plant species have rarely been characterized (but see Westerbergh and Saura 1994; Lau et al. 2008). Not only were florivore damage and pollinator visitation rates altered by soil habitat, but pollinator assemblage was also more diverse for *M. guttatus* in non-serpentine soils (Tables 5, 6) indicating that serpentine soil influences the quantity and perhaps the quality of plant-animal interactions.

Mimulus guttatus on serpentine had reduced floral display (i.e., smaller flowers, fewer flowers per inflorescence and shorter inflorescences), and our common garden reciprocal soil transplant experiment confirmed the direct effect of serpentine soil on flower size and inflorescence height (Fig. 4) in response to nutrient limitation. Interestingly, plants responded similarly to soil treatments in terms of morphology and tissue chemistry, regardless of the soil type they originated. In addition to similar morphological and chemical responses, all experimental plants survived equally well on both soil types, regardless of whether they were collected from serpentine or non-serpentine populations (χ^2=0.14, df=1, P=0.71). This suggests a lack of adaptation to soil chemistry for these serpentine/non-serpentine populations of *M. guttatus*, which may be explained by high levels of gene flow between populations growing in

different soil environments (Sambatti and Rice 2006). And if our survival data is indicative of total fitness then our findings are in contrast to others that have found evidence of local adaptation and ecotypic differentiation for other serpentine tolerant plant species (e.g., *Collinsia sparsiflora*; Wright et al. 2006). However, other studies have documented the importance of phenotypic plasticity for serpentine tolerant *M. guttatus* (Murren et al. 2006), and it is clear from our data that soil-induced changes in plant morphology have consequences for plant-pollinator and plant-florivore interactions in this species. While flower size varied across serpentine and non-serpentine populations, pollinator visitation was greater in plots with larger flowers within both serpentine and non-serpentine sites (Table 4), and pollinators responded in terms of increased visitation to larger flowers within experimental arrays (Table 10). Additionally, the number of open flowers per inflorescence and inflorescence height altered plant-florivore interactions, as plants with more flowers and taller inflorescences experienced greater levels of florivore damage in natural populations (Table 7). Therefore, indirect effects of soil environment on pollinator visitation and florivore damage may affect plant-animal dynamics in plant species expressing phenotypic plasticity across multiple environments. Phenotypic plasticity due to environmental heterogeneity is likely an important, yet understudied, mechanism altering the evolution of plant-animal interactions (Fordyce 2006).

Traditionally, studies of plant-herbivore interactions have focused on the role of plant secondary compounds in influencing levels of herbivore damage (reviewed in Mithöfer and Boland 2012). However, primary metabolites may be equally important in affecting herbivore damage (e.g., Alonso and Herrera 2003). Studies of herbivory of plants growing on serpentines have often focused on toxic elements in the soil, such as the heavy metal Ni in hyperaccumulating species (Boyd et al. 1994; Martens and Boyd 1994; Boyd and Moar 1999).

Our work with *M. guttatus*, a serpentine tolerant species, did not reveal significant differences in Ni concentrations in floral tissues of plants growing in serpentine vs. non-serpentine soils (Fig. 2) yet there were tendencies for higher florivore damage on non-serpentine grown plants in both natural and experimental settings (Figs. 3, 5). Because plants growing in serpentine vs. non-serpentine soils had distinct chemical profiles (Fig. 2), these findings suggest that other metals (e.g., Mg) or primary metabolites such as Ca, P and K may be just as likely to alter herbivore feeding as the toxic metal Ni present in serpentines. While the effects of primary metabolites on the growth of insect herbivores is well studied, the influence of primary metabolites on herbivore choice is less understood (Joern et al. 2012). Furthermore, previous work has suggested that herbivores may respond to ratios of elements, rather than single element concentrations, which may be the case in serpentine plants as the chemical profiles of serpentine vs. non-serpentine tissues differed in multiple elements. For example, Beanland et al. (2003) manipulated the ratios of B, Zn and Fe present in diets fed to herbivores, and found that herbivore development could not be described as a linear response to any one element, but instead depended upon ratios of these elements. As several elements varied in the floral tissues of *M. guttatus* in this study, it is reasonable to suspect that *M. guttatus* florivores are also responding to multiple element variation. Our work shows that soil generalist, non-hyperaccumulating plants may display variation in the magnitude of plant-florivore interactions across multiple environments, and that this variation is largely explained by the direct effect of soil chemistry on floral tissue chemistry.

We did not find evidence of a direct effect of flower chemistry on pollinator visitation, which may be due to one of several factors. For example, our tissue analysis was based on whole flowers, therefore we do not know if the soil environment alters pollen or nectar chemistry for *M. guttatus*, or rather strictly perianth tissues. Furthermore, *M. guttatus* is not known to

produce large volumes of nectar (Robertson et al. 1999), thus flower visiting insects may not have been exposed to chemically variable resources when visiting serpentine vs. non-serpentine *M. guttatus*, particularly if pollen chemistry is unaffected by soil environment. It is also possible that pollinating insects are less affected by changes in tissue chemistry relative to herbivorous insects, as visitation by bees has been shown to be unaffected by high concentrations of trace elements in floral rewards (e.g., Se: Hladun et al. 2013). Other studies, however, have shown that the presence of Ni in nectar can decrease visitation by bumblebees (Meindl and Ashman 2013), suggesting soil chemistry may alter biotic interactions more so than previously thought.

It is important to consider whether differences observed in plant-pollinator and plant-florivore interactions across serpentine and non-serpentine habitats translate into differences in individual fitness of plants in natural populations. Provided that plants are pollen-limited, which is common for many flowering plants (reviewed in Knight et al. 2005), pollinator visitation is generally considered a good proxy of fitness as higher visitation rates often translate into higher seed and fruit production (e.g., Ghazoul 2005). However, in nutrient-limited environments, like serpentine, limited resource availability may preclude any added benefit of increased pollinator visitation towards individual fitness (Asikainen and Mutikainen 2005). Additionally, while corolla damage by florivores can decrease pollinator visitation (e.g. Botto-Mahan et al. 2011), florivore damage to structures that house the gametes, such as anthers and pistils, may have greater consequences for plant fitness (McCall and Irwin 2006; Hargraves et al. 2009), especially if plant tolerance to herbivory is low (Strauss and Agrawal 1999). To fully appreciate the evolutionary consequences of abiotic-mediated changes in plant-animal interactions, studies are needed that document differential fitness of soil-generalist plants across different environments and that tie this directly to altered plant-animal interactions.

Our study shows that soils have both direct and indirect effects on how plants interact with animal mutualists and antagonists. We demonstrate that soils can affect these plant-animal interactions more generally than previously thought, i.e., in addition to affecting plant-insect interactions of metal hyperaccumulators, even those of non-accumulating soil generalist species are affected. As such, plant species that occur in a variety of substrates may differ in both the quality (e.g., visits by effective pollinators) and quantity (e.g., number of pollinator visits) of plant-animal interactions across soil types. Soil chemistry may therefore be an important geographic variant that contributes to altered interactions, leading to small scale spatial mosaics with the potential to influence evolutionary dynamics between plants and animals (Thompson 1999).

Table 1. Chemical composition of serpentine and non-serpentine soils collected from field sites at the McLaughlin Reserve, Lower Lake, CA. Chemical analysis of soil samples completed via ICP-MS by ALS Minerals, Reno, NV, USA.

Soil Type	Al (%)	Ca (%)	Co (ppm)	Cr (ppm)	Fe (%)	K (%)	Mg (%)	Ni (ppm)	P (%)	Zn (ppm)
Non-serpentine	2.33	0.75	34.95	165.5	3.57	0.22	3.25	319.5	0.0575	91.35
Serpentine	1.69	0.42	100.45	670.5	5.565	0.135	13.875	1670	0.0295	154

Table 2. Results from mixed models ANOVA on the effects of serpentine vs. non-serpentine soil (site soil) and year (2010, 2011) on inflorescence height, corolla width and number of open flowers per inflorescence of *M. guttatus* plants in natural populations. Significance of fixed effects at $P \leq 0.05$ is noted with daggers and at $P \leq 0.01$ with asterisks.

		Display Trait		
		Inflorescence height	Corolla width	Number of open flowers per inflorescence
Source of variation	df (Num., Den.)	*F*	*F*	*F*
Site soil type	1, 2	80.96†	175.59*	187.09*
Year	1, 159	1.13	29.18*	4.90†

Table 3. Results from mixed models ANOVA on the effects of serpentine vs. non-serpentine soil (Site soil type) on element concentration of *M. guttatus* flowers collected from natural populations. Bold *F*-values are those significant after Bonferroni correction.

		Element									
		Macronutrients				Micronutrients				Other elements	
		Ca	Mg	P	K	Fe	Ni	Zn	B	Na	Al
Source of variation	df (Num., Den.)	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*
Site soil type	1, 2	**131.08**	3.08	70.02	7.74	5.34	0.64	2.89	1.18	15.67	6.3

Table 4. Mixed model ANCOVA of pollinator visitation rate (visits/flower/hour) to *M. guttatus* plants growing at serpentine vs. non-serpentine sites (Site soil) in two years (2010, 2011). Time of day of observation, the number of heterospecific flowers within plots, percent bare soil within plots, and plot-level means of corolla width, open flowers per inflorescence, and inflorescence height were included as covariates. Significance of fixed effects at $P \leq 0.05$ is noted with daggers and at $P \leq 0.01$ with asterisks

Source of variation	df (Num., Den.)	*F*
Site soil type	1, 2	17.35†
Year	1, 154	8.29*
Corolla width	1, 154	9.23*
Open flowers per inflorescence	1, 154	0.32
Inflorescence height	1, 154	0.06
Time of day	1, 154	3.76
Number of heterospecific flowers	1, 154	1.19
% bare soil	1, 154	0.10

Table 5. Pollinators observed at non-serpentine and serpentine *M. guttatus* populations over the course of the two-year study period. In 2011, pollinators were further differentiated by species (bees) or family (beetles) within larger categories and the number of taxa observed are given in parentheses (e.g., 6 different large bee species were observed in non-serpentine populations). The following families were recorded within each category: large bee- Anthophoridae, Apidae, Megachildae; medium bee- Anthophoridae, Halictidae, Megachilidae; small bee- Anthophoridae, Halictidae, Megachilidae; beetle- Buprestidae, Chrysomlidae, Cleridae, Melyridae.

	2010		2011	
Pollinator Group	Number of Individuals Observed on Serpentine	Number of Individuals Observed on Non-serpentine	Number of Individuals (Taxa) Observed on Serpentine	Number of Individuals (Taxa) Observed on Non-serpentine
Large bee	5	77	13 (3)	84 (5)
Medium bee	36	53	59 (6)	185 (10)
Small bee	29	34	35 (7)	66 (9)
Beetle	1	39	5 (2)	71 (4)
TOTAL	71	203	112 (18)	406 (27)

Table 6. Bee species collected on *M. guttatus* flowers from non-serpentine (24) and serpentine (16) populations in 2011. The symbol 'X' implies a given species was collected.

Bee Species	Collected at Non-serpentine	Collected at Serpentine
Anthidium edwardsii		X
Apis mellifera	X	X
Ashmeadiella australis		X
Ashmeadiella salviae	X	
Bombus vosnesenskii	X	
Calliopsis trifolii		X
Ceratina nanula	X	
Ceratina punctigena	X	
Ceratina sequoiae	X	
Ceratina tejonensis	X	X
Ceratina timberlakei	X	X
Chelostoma minutum	X	
Diadasia sp. 1	X	
Dialictus sp. 1	X	
Dialictus sp. 2	X	X
Dialictus sp. 3	X	
Dialictus sp. 4		X
Evylaeus sp. 1	X	X
Halictus ligatus		X
Heriades sp. 1	X	
Hoplitis hypocrita	X	
Hoplitis producta	X	X
Hoplitis sambuci	X	X
Lasioglossum sp. 1		X
Osmia sp. 1	X	
Osmia sp. 2	X	X
Osmia sp. 3	X	
Osmia sp. 4	X	
Osmia sp. 5	X	X
Protosmia rubifloris	X	X

Table 7. Results from mixed model ANOVA of florivore damage (percentage of flowers with florivore damage per inflorescence) to *M. guttatus* plants from natural serpentine vs. non-serpentine populations (site soil) in two years (2010, 2011). Plot-level means of corolla width, open flowers per inflorescence, and inflorescence height were included as covariates. Significance of fixed effects at $P \leq 0.05$ is noted with daggers and at $P \leq 0.01$ with asterisks.

Source of variation	df (Num., Den.)	*F*
Site soil type	1, 2	6.32
Year	1, 146	0.73
Flower number	1, 146	5.46†
Corolla width	1, 146	1.51
Inflorescence height	1, 146	15.57*

Table 8. Results from the mixed models ANOVA on floral display traits (inflorescence height, corolla width, and number of open flowers per inflorescence) of *M. guttatus* plants in reciprocal transplant experiment. Experimental and source population soils are serpentine and non-serpentine. Asterisks ($P \leq 0.01$) indicate significant fixed effects.

		Display Trait		
		Inflorescence height	Corolla width	Number of open flowers per inflorescence
Source of variation	df (Num., Den.)	*F*	*F*	*F*
Experimental soil type	1, 160	13.35*	207.97*	2.83
Source population soil type	1, 2	0.03	0.48	0.89
Experimental soil type*Source population soil type	1, 160	0.17	1.78	0.15

Table 9. Results from the mixed models ANOVA on element concentrations of *M. guttatus* plants in reciprocal transplant experiment. Experimental soil types and source population soil types are serpentine and non-serpentine. Bold *F*-values are those significant after Bonferroni correction.

		Element									
		Macronutrients				Micronutrients				Other elements	
		Ca	Mg	P	K	Fe	Ni	Zn	B	Na	Al
Source of variation	df (Num., Den.)	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*	*F*
Source population soil type	1, 2	2	0.05	0.38	0.65	0.06	0.3	0.96	0.04	0.72	0.01
Experimental soil type	1, 134	**63.38**	**63.88**	**31.28**	**15.66**	**8.09**	0.06	1.7	3.52	5.22	5.45

Table 10. Results from mixed model ANCOVA of pollinator visitation rate (visits/flower/hour) to experimental arrays of *M. guttatus* inflorescences collected from serpentine vs. non-serpentine sites (source population soil) and presented to pollinators at serpentine or non-serpentine sites (site soil). Corolla width of experimental plants was included as a covariate. Significance of fixed effects at $P \leq 0.05$ is noted with daggers and at $P \leq 0.01$ with asterisks.

Source of variation	df (Num., Den.)	*F*
Time of day	1, 182	3.18
Site soil type	1, 2	20.35†
Source population soil type	1, 2	1.81
Corolla width	1, 182	67.48*

Table 11. Results of the generalized mixed model of florivore damage score to *M. guttatus* plants collected from serpentine vs. non-serpentine sites (source population soil) and presented to florivores at serpentine or non-serpentine sites (site soil). Over the course of three weeks, one array (*N*=16-20 plants) was monitored weekly for florivore damage at each site. Inflorescence height and corolla width were included in the model as covariates; plus sign indicates *P*≤0.06.

Source of variation	df (Num., Den.)	*F*
Site soil type	1, 2	3.14
Source population soil type	1, 2	16.59+
Inflorescence height	1, 42	0.02
Corolla width	1, 42	1.69
Week	2, 42	0.43

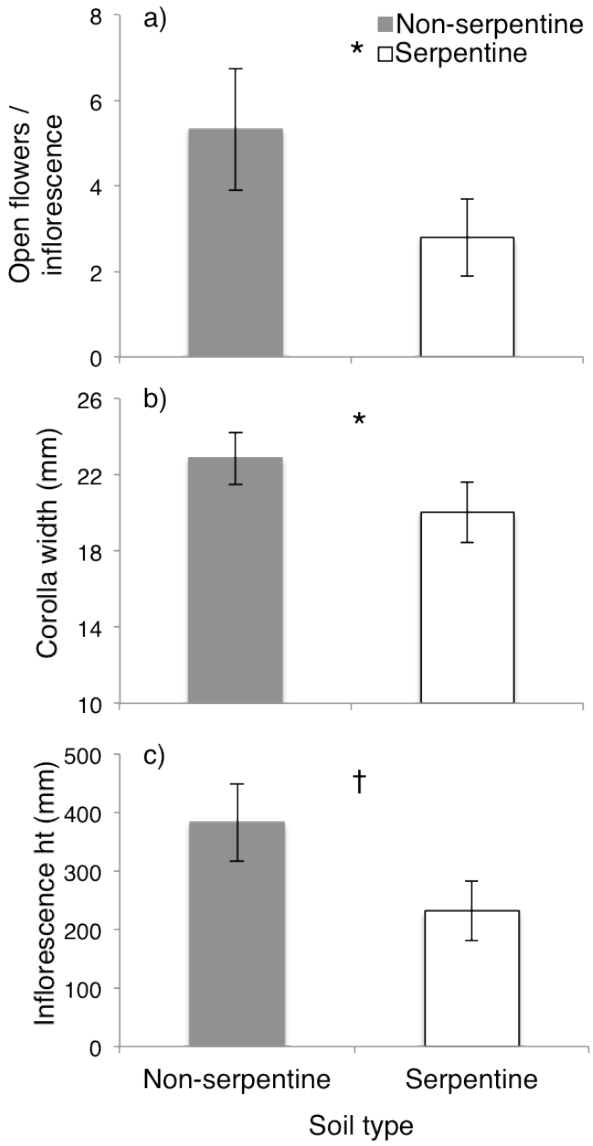

Figure 1. Comparison of a) number of open flowers per inflorescence, b) corolla width, and c) inflorescence height of *M. guttatus* plants growing in natural non-serpentine and serpentine sites. Bars are means (± SE; *N*=82 per soil type); daggers (*P*≤0.05) and asterisks (*P*≤0.01) indicate a significant soil type effect.

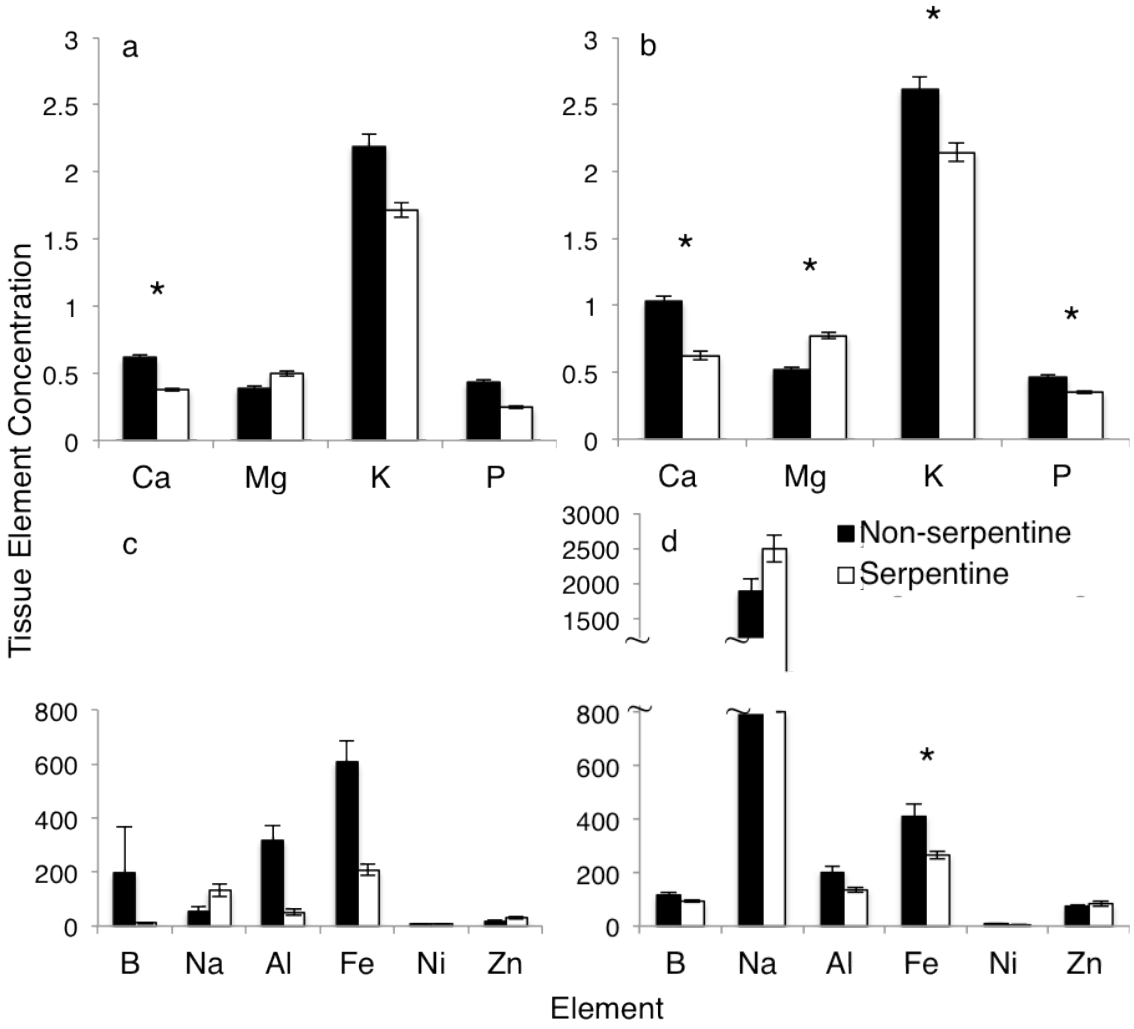

Figure 2. Differences in flower concentration of (a, b; %) macronutrients and (c, d; ppm) micronutrients and beneficial elements of *M. guttatus* plants growing in (a, c) natural populations and (b, d) experimental soils. Bars are means (± SE; *N*=82 per natural soil type; *N*=83-88 per experimental soil type); asterisks (*P*≤0.005) indicate a significant soil type (serpentine vs. non-serpentine) effect.

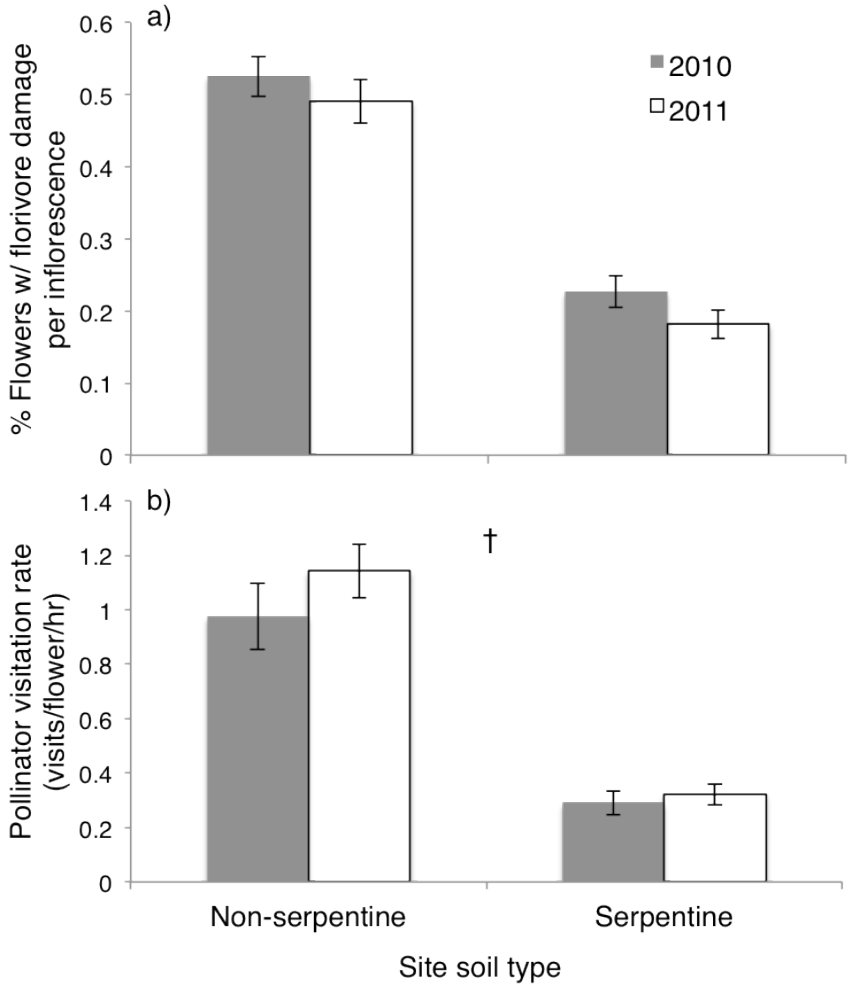

Figure 3. Pollinator visitation rates (visits/flower/hour) and florivore damage (% of flowers damaged per inflorescence) at non-serpentine and serpentine populations of *M. guttatus* in 2010 and 2011. Bars are means (± SE; *N*=82 per soil type); daggers (*P*≤0.05) indicate a significant site soil type effect.

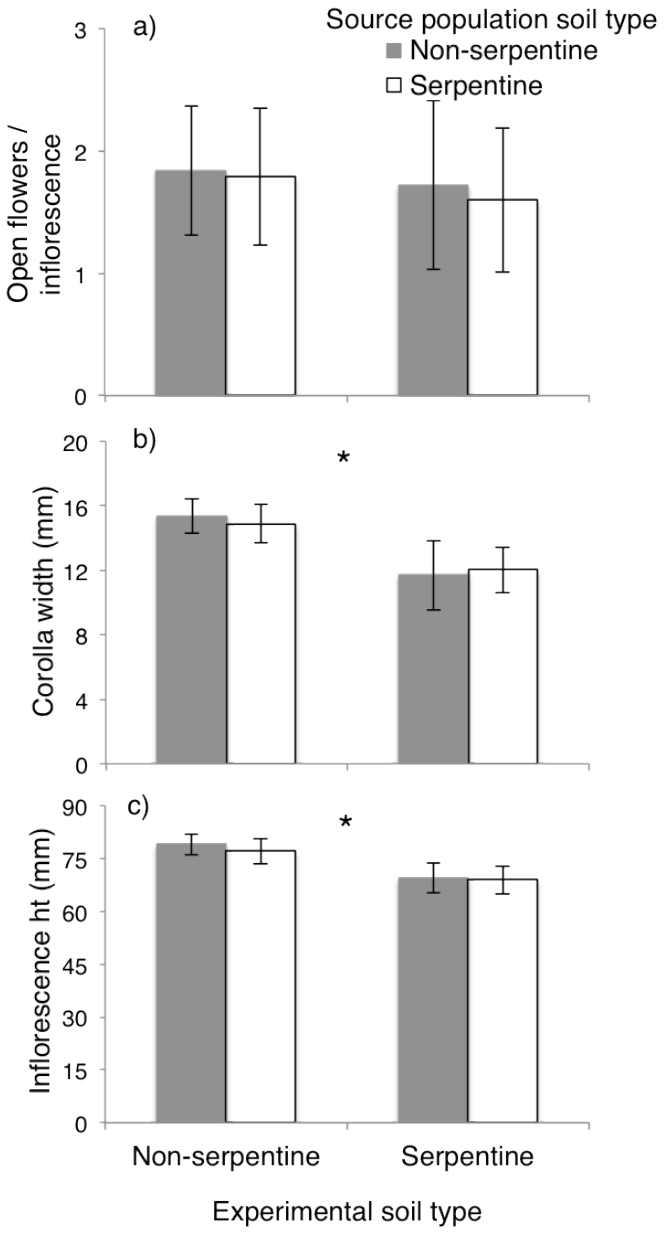

Figure 4. Effect of experimental serpentine soil on a) number of open flowers per inflorescence, b) corolla width, and c) inflorescence height of *M. guttatus* plants in reciprocal soil transplant experiment. Bars are means (± SE, *N*=83-88 per experimental soil type); asterisks (*P*≤0.01) indicate a significant experimental soil type (serpentine vs. non-serpentine) effect.

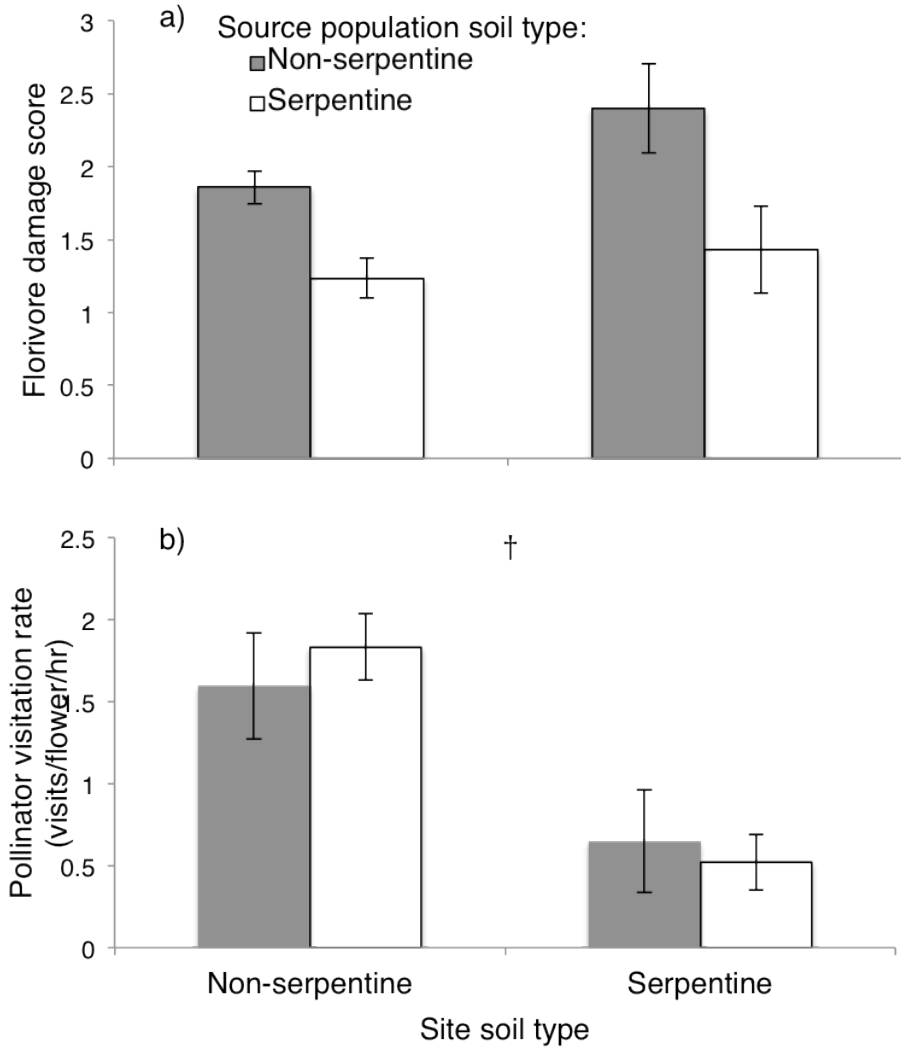

Figure 5. Effects of site soil type and source population on a) florivore damage score and b) pollinator visitation rates (visits/flower/hour) to potted *M. guttatus* in experimental arrays. Bars are means (± SE, *N*=96-104 plants per site soil type); daggers (*P*≤0.05) indicate a significant site soil type effect.

3.0 NICKEL ACCUMULATION IN LEAVES, FLORAL ORGANS AND REWARDS VARIES BY SERPENTINE SOIL AFFINITY

3.1 INTRODUCTION

Edaphic factors, such as soil texture, depth and chemical composition, are a primary force in shaping the distributions of plant species (Silvertown, 2004; Toledo *et al*., 2012; Dubuis *et al*., 2013). While many plant species can be found growing in a variety of habitats, some species become entirely restricted to a particular soil type (i.e. edaphic endemics; Macnair and Gardner, 1998; Rajakaruna, 2004). Serpentine soils, which can be found on every continent (Brooks, 1987), provide one of the most remarkable examples of plant adaptation to atypical soils (O'Dell and Rajakaruna, 2011) and geographic regions containing serpentine often harbor numerous endemic species (Brooks, 1987; Safford *et al*., 2005; Anacker 2011). Serpentine-derived soils represent a nutritionally stressful growing environment for most plants because of a low Ca:Mg ratio, deficiency of essential nutrients (e.g. N, P, K), and high levels of potentially phytotoxic heavy metals (e.g. nickel [Ni], cobalt, chromium; Brooks, 1987; Brady *et al*., 2005; Kazakou *et al*., 2008). While comparisons of plant tissue chemistry between endemic and non-endemic species can provide insight into the physiological features of edaphic endemics (Palacio *et al*., 2007), it is unclear whether soil affinity (i.e. endemic vs. non-endemic) affects plant tissue chemistry for serpentine plant species. For example, if endemic species are specifically adapted

to the abiotic stresses of serpentine soil, then they might be better able to acquire limiting resources and/or exclude phytotoxic elements from the soil than non-endemic species. Considering that species span a gradient of affinity to serpentine soils, with some only occasionally found on serpentines (i.e. 'indifferent', ≤45% occurrences on serpentines), some commonly found either on or off serpentines (i.e. 'indicator', ~55-64% occurrences on serpentines) whereas others are entirely restricted to serpentines (i.e. 'endemic', ≥95% occurrences on serpentines) (Safford *et al*., 2005), serpentine soils provide an ideal system to test whether soil affinity affects tissue chemistry.

The elevated heavy metal concentrations, in particular, in serpentine soils are thought to drive levels of plant adaptation (Lazarus *et al*., 2011), as non-adapted species lack physiological mechanisms to avoid metal toxicity (e.g. via metal exclusion or sequestration with chelating agents; Kazakou *et al*., 2008). However, of the few studies examining the differences in metal accumulation between endemic and non-endemic species, some have found decreased shoot metal accumulation in endemic species (Nagy and Proctor, 1997; Burrell *et al*., 2012) while others have found no such difference (Fiedler, 1985; Lee and Reeves, 1989). Plant response to the heavy metal Ni found in serpentines is relatively well studied (Kazakou *et al*., 2008), and provides an excellent model to test whether endemic vs. non-endemic species vary in heavy metal accumulation. While some plants require Ni in trace quantities as an active component of the enzyme urease (Welch, 1981), it is generally considered toxic to plants and is implicated in causing abnormal vegetative growth, necrosis and chlorosis of leaves, and inhibiting photosynthesis (reviewed in Yusuf *et al*., 2011). Furthermore, Ni is known to negatively impact aspects of plant reproduction for non-hyperaccumulators, such as decreasing pollen germination (Tuna *et al*., 2002; Breygina *et al*., 2012) and seed production (Malan and Farrant, 1998) when

plants not adapted to elevated Ni conditions are grown in them. However, whether Ni is accumulated into reproductive organs, such as anthers and pistils, has only been studied in one serpentine plant species that is a known Ni hyperaccumulator (i.e., accumulates >1,000 ppm Ni [*Streptanthus polygaloides*]; Meindl and Ashman, 2014; Sánchez-Mata *et al*., 2014). While Ni hyperaccumulators are often specialized to serpentines (Reeves and Baker 2000), they represent an extreme minority, in both number of taxa and plant-soil interactions, of serpentine endemic plant species (Reeves, 2006; Anacker, 2011). Beyond these rare, yet relatively well-studied, hyperaccumulators (Reeves, 2006; Gall and Rajakaruna, 2013), it is largely unknown whether the vast majority of serpentine species exhibit significant variation in Ni accumulation into aboveground organs. Therefore, studies are needed that focus on metal uptake for non-hyperaccumulating species to determine more general patterns of metal uptake or exclusion across serpentine plant species. Furthermore, it is unknown whether most serpentine plants accumulate Ni into pollen grains, despite evidence that plants growing in soils contaminated by metals via human activities can accumulate them into pollen (Moroń *et al*., 2012). Metals in pollen could reduce germination (citations above; Mohsenzadeh *et al*., 2011; Yousefi *et al*., 2011*a*) or pollinator attraction (Meindl and Ashman, 2014), and Ni accumulation in nectar can affect pollinator foraging (Meindl and Ashman, 2013; Meindl and Ashman, 2014). Thus, a first and necessary step towards understanding the reproductive consequences of growth on serpentine soil is documenting metal concentrations of reproductive organs and floral rewards of non-hyperaccumulating serpentine plants, as well as determining whether or not non-hyperaccumulating endemic species are better able to avoid potentially deleterious effects of metals by excluding them from reproductive organs than non-endemics. However, explicit

experimental comparisons of metal accumulation across a range of species that vary in serpentine affinity, as well as across a range of vegetative and reproductive organs, are lacking.

To test these ideas, we grew seven species of plants from the Brassicaceae family that varied in serpentine soil affinity, but are not considered metal hyperaccumulators, in either control soils or soils supplemented with Ni to determine whether serpentine endemic and non-endemic plants differ with respect to Ni uptake (i.e., accumulate lower or similar concentrations of Ni into leaves, reproductive organs and rewards compared to non-endemic species, respectively). Using Ni as a model for plant response to serpentine heavy metals in general, we answered these questions: (1) Do serpentine endemic and non-endemic species differ in terms of Ni uptake into (i) leaves, (ii) pistils, (iii) anthers, and/or (iv) nectar? (2) Do serpentine endemics and non-endemics differ in the relative concentrations of Ni in vegetative vs. reproductive organs? (3) Is Ni incorporated into pollen grains in any of these species?

3.2 METHODS

3.2.1 Study system

Plants in the Brassicaceae (mustard family) are well represented on California serpentine soils (Safford *et al*., 2005), including the seven species used here that differ in serpentine affinity from strictly endemic (i.e. ≥95% occurrence on serpentine) to indifferent to serpentine soils (i.e. ≤45% occurrence on serpentine soils): endemic: *Streptanthus morrisonii, S. breweri* var. *breweri*; indicator: *S. glandulosus* ssp. *glandulosu*s, *S. tortuosus*; indifferent: *Hirschfeldia incana*,

Erysimum capitatum var. *capitatum*, *Boechera breweri* (Table 12). To assign serpentine affinity scores to taxa, we follow the nomenclature used in Safford et al. (2005), however, in this work we follow the recent revised nomenclature for two taxa (i.e., *S. tortuosus* var. *suffrutescens* [now *S. tortuosus*] and *Arabis breweri* [now *Boechera breweri*]; Baldwin et al. 2012). All are spring flowering, insect pollinated herbaceous annuals or perennials that occur in North America, with four taxa being restricted to California (Table 12). Seeds from each taxon were bulk-collected from a single population per species in the summer of 2012.

3.2.2 Experimental design

Twenty plants per species (Total $N = 140$) were grown at the University of Pittsburgh in the fall of 2012. Seeds were subjected to a 4°C cold and dark treatment for two weeks prior to planting. Two weeks after germination, seedlings were transplanted to 27 cm^3 'rocket' pots (Deepots, Stuewe and Sons, Inc., Tangent, OR, USA) filled with standard potting soil (Fafard #4, Sun Gro Horticulture, Agawam, MA, USA) and six Nutricote® NPK 13-13-13 time-release fertilizer pellets (Arysta LifeScience Corporation, New York, NY, USA). One month after transplanting, all perennials (*S. morrisonii, E. capitatum* var. *capitatum, S. tortuosus, B. breweri*) received a 4°C cold treatment for one month at 8D:16N. Subsequently, these perennials and the annuals (*S. breweri* var. *breweri, H. incana, S. glandulosus* ssp. *glandulosus*), were grown under controlled conditions of 12D:12N, between 70-80° F, until flowering.

One month after potting (annuals), or one week after cold treatment (perennials), soil treatment solutions were applied to each plant weekly: either (1) Ni-supplemented (40 mL of 400 ppm Ni nitrate ($Ni(NO_3)_2$-$6H_2O$) solution) or (2) control (40 mL of ammonium nitrate (NH_4NO_3)

solution to compensate for 190 ppm nitrogen applied to plants in the Ni-supplemented soil treatment). This reflects a natural level of Ni, as serpentine soils contain bioavailable fractions of Ni ranging from 50 ppm to 500 ppm (e.g. Chardot *et al*., 2005; L'Huillier and Edighoffer, 1996). Soil treatments were conducted for 4-18 weeks, depending on time to flower. All plants were watered as needed.

3.2.3 Organ/reward collection and chemical analysis

Three organs (leaves, pistils, anthers) and two floral rewards (pollen, nectar) were collected from individual plants. A single fully developed leaf from the basal rosette was collected from each plant after four soil treatment applications. Pistils, anthers, and nectar were collected from the first 5-15 flowers produced per plant. To collect nectar from several flowers per plant we folded a circular piece of filter paper (Whatman® Grade 1, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) in half and touched it to the floral nectaries in a circular pattern. Nectar volume was determined via Baker's (1979) spot-staining method, as described in Kearns and Inouye (1993). The measured diameter (mm) of each nectar spot was compared to a standard table that relates spot diameter to nectar volume (uL). This technique is valid for nectars with sugar concentrations ranging from 10-50% and nectar spot diameters ≤ 12 mm, which is true for many Brassicaceae (e.g. Masierowska, 2003; Nedić *et al*., 2013) including those in our study. Pistils and anthers were dissected from freshly-opened whole flowers using forceps. While leaves were collected from every plant (N = 10 per species-soil treatment), some plants (N = 5) never flowered and thus 7-10 plants were sampled per species-soil treatment for floral organs and

rewards. In addition, pollen was collected from two plants per species-soil treatment from an independent set of mature anthers.

Prior to chemical analysis, leaves and pistils were rinsed with diH_2O and dried at 60°C for 48 hours. Anther, pollen, and nectar samples were allowed to air dry for 48 hours in microcentrifuge tubes. Samples were weighed to the nearest 0.0001 g on a AE200 Mettler® analytical balance (Mettler-Toledo, LLC, Columbus, OH, USA) and microwave digested in 2-4 mL of trace metal grade HNO_3 and brought to a final volume of 12-14 mL with MilliQ (Millipore, Bedford, MA, USA) H_2O. Concentration of Ni is reported as ppm in organs and pollen (i.e., mg kg^{-1}) and nectar (i.e., uL L^{-1}) and was determined via Inductively Coupled Plasma Mass Spectrometry (ICP-MS, NEXION 300X, PerkinElmer, Waltham, MA, USA) at the University of Pittsburgh (for details see Meindl and Ashman, 2014).

3.2.4 Statistical analysis

All statistical analyses were conducted in SAS (version 9.3; SAS Institute Inc., Cary, NC, USA). To evaluate the effect of soil treatment, serpentine habitat affinity, and organ/reward type on plant Ni concentration, mixed-model ANCOVA was conducted (PROC MIXED). The model included the fixed effects of soil treatment (Ni supplement, control), serpentine habitat affinity (endemic, indicator or indifferent), organ/reward type (leaves, pistils, anthers or nectar), and their interactions, and random factors of individual and species, where species was nested within serpentine habitat affinity (Table 1). The number of Ni applications to the soil was included as a covariate ('application number'). Denominator degrees of freedom for *F*-tests were determined using the Kenward-Roger approximation, which is preferred for small sample sizes and

unbalanced data (Littell et al. 2002). For Ni-treated plants only, we used pre-planned contrasts to determine whether endemic species 1) incorporated less Ni than indicator/indifferent species in organs/rewards, and 2) displayed lower concentrations of Ni in reproductive organs (anthers, pistils) relative to leaves than indicator/indifferent species using the CONTRAST option. We used a student's *t*-test (PROC TTEST) to determine if pollen Ni concentration was higher in Ni-treated plants than controls. For all analyses, Ni concentrations were natural-log transformed to improve normality of residuals. Back transformed lsmeans (and 95% confidence intervals) of Ni concentrations are presented for clarity.

3.3 RESULTS

Soil Ni treatment was effective, as mean Ni concentrations in Ni-treated plants were 16 times higher than control plants across all organs and nectar (46.5 ppm vs. 3.00 ppm), but the effect of habitat affinity on Ni in plant tissue was dependent on both soil treatment and organ/reward type (Habitat Affinity x Soil Treatment x Organ/Reward Type: $P<0.05$; Table 13; Fig. 6). Within the Ni soil treatment, endemic species had lower Ni concentrations in leaves and pistils compared to both indicator and indifferent species (leaves-- endemic: 39.0 ppm; indicator and indifferent: 62.2 ppm; pistils-- endemic: 55.3 ppm; indicator and indifferent: 99.5 ppm; Table 13). However, endemic species did not have significantly lower Ni concentrations compared to indicator/indifferent species in either anthers (endemic: 72.9 ppm; indicator/indifferent: 99.5 ppm; Table 13) or nectar (endemic: 13.1 ppm; indicator/indifferent: 12.8 ppm; Table 13). Furthermore, within the Ni soil treatment, indifferent species had Ni concentrations in

anthers/pistils (126.5 ppm) that were twice as high as that in leaves (62.8 ppm; $P<0.0001$, Table 13). Conversely, we did not detect a difference between endemic and indicator species with respect to concentrations of Ni in reproductive relative to vegetative organs (anthers/pistils vs. leaves: endemic 59.7 vs. 39.0 ppm; indicator: 72.2 vs. 66.7 ppm; Table 13).

Across all species, mean Ni concentrations in pollen were 10 times higher in Ni-treated plants than controls (50.9 ppm vs. 5.7 ppm; $t = -5.33$; df = 26; $P < 0.0001$). Nickel concentration of pollen from Ni-treated plants was highest for indicator species (59.1 ppm), followed by endemic (44.7 ppm) and indifferent (38.9 ppm) species.

3.4 DISCUSSION

Serpentine endemic species incorporated Ni in lower concentrations in leaves and reproductive organs compared to indicator/indifferent species, suggesting that these species may be better adapted to the Ni-rich serpentine soil environment than the indicator/indifferent species studied. However, the magnitude of this difference depended on organ type, as endemics incorporated significantly less Ni into leaves and pistils, but not anthers, compared to indicator/indifferent species. Furthermore, while Ni exclusion is one possible mechanism to limit toxicity, effective sequestration in leaves is another (Yusuf *et al.*, 2011). Species indifferent to serpentine had higher Ni concentrations in reproductive organs relative to leaves, whereas endemic and indicator species had similar Ni concentrations across all organs, again suggesting that plant species not regularly associated with serpentines do not possess mechanisms to limit uptake of Ni into reproductive organs.

In the present study, serpentine endemic Brassicaceae exhibited the greatest degree of Ni exclusion, particularly in the leaves and pistils. Similarly, DeHart *et al*. (in review) found that while there was no difference in Ni concentrations between field-collected leaves, flowers or seeds of serpentine endemic and non-endemic species from the plant families Fabaceae, Phrymaceae, and Ranunculaceae (possibly due to low levels of phytoavailable Ni in soils at their study sites), endemic species had significantly lower concentrations of the heavy metal cobalt across all organs than non-endemics (DeHart *et al*., in review). Furthermore, studies of edaphic endemics in other soil environments also suggest endemic plants may be specialized to their respective soil environment relative to non-endemics. By comparing leaf tissue chemistry of plant species that were either endemic or non-endemic to gypsum soils, which are high in sulfate ions and low in several macronutrients, Palacio *et al*. (2007) concluded that many gypsum soil endemic plants were more efficient at extracting limiting nutrients (e.g. N, P) from gypsum soils relative to non-endemic species, though range size also played a role in the level of specialization observed. Taken together, these findings suggest that many edaphic endemics may be physiologically better suited to their respective soil environments, e.g., by excluding heavy metals or acquiring limiting nutrients, than non-endemics. Our results contribute to ideas that support the specialist model of edaphic endemism (Meyer, 1986; Palacio *et al.*, 2007) rather than the refuge model, in which endemics are not specifically adapted a particular soil type (Gankin and Major, 1964), although studies of tissue chemistry would have to be coupled with measures of fitness to confirm this idea. It is important to recognize, however, that we intentionally excluded from our study serpentine soil endemics that are known to hyperaccumulate Ni, such as *S. polygaloides*. These species represent an exception to plant metal accumulation by serpentine soil endemics, rather than the rule (Reeves, 2006; Anacker, 2011), and thus predictions relating

to heavy metal accumulation would clearly differ when considering metal hyperaccumulating taxa. However, because metal hyperaccumulation may impart chemical defenses to plants (Rascio and Navari-Izzo, 2011) and thus may impact plant fitness, documenting metal hyperaccumulation into reproductive organs and rewards in these species may provide valuable insight into the potential adaptive value of metal hyperaccumulation (Boyd and Martens, 1992) and warrants additional study. In fact, recent experimental evidence suggests that two Ni hyperaccumulating taxa concentrate Ni in both vegetative and reproductive organs (*S. polygaloides* and *Noccaea fendleri*; Meindl *et al.*, in review). Furthermore, other chemical aspects of serpentine soils besides high Ni concentrations, such as low Ca:Mg ratios, may be equally important drivers of plant adaptation to the serpentine soil environment (Brady *et al*., 2005; O'Dell and Rajakaruna, 2011; DeHart *et al*., in review). Further comparisons of both macronutrient (e.g., Ca, Mg, N, K, P) and heavy metal (e.g., Ni, Co, Cr) concentrations between tissues of endemic and non-endemic species will provide a more comprehensive view of plant adaptation to serpentine soils.

Interestingly, Ni accumulation was, on average, higher in reproductive organs compared to leaves across all species in this study, corroborating similar findings of increased metal accumulation in flowers relative to leaves (Severne, 1974; Gabbrielli *et al*., 1997). However, our work suggests that plants restricted to soils with elevated metals generally have lower metal concentrations in floral organs relative to plants not restricted to such soils. This pattern suggests a cost to floral metal accumulation, which could relate to decreased reproductive success via negative impacts on pollen and ovule viability, as well as seed and fruit production (Maestri *et al*., 2010). Indeed, recent studies suggest that floral metal accumulation can decrease both pollen and ovule viability due to developmental abnormalities in anthers and ovaries (Yousefi *et al*.,

2011*a*, *b*). While we show that Ni is incorporated into pistils, anthers, pollen, and nectar, specifically, further work elucidating the effects of floral Ni accumulation on ovule and pollen viability and reproductive success in natural populations will provide necessary information towards understanding the adaptive value of both metal exclusion and metal accumulation. For example, pollen germination for some plant species known to accumulate high concentrations of the metalloid selenium is actually improved by increasing concentrations of selenium in pistils (Prins *et al*., 2011). However, the effects of floral metal accumulation on plant fitness for serpentine species are unknown, though experiments testing these effects are currently underway (Meindl and Ashman, unpubl. res.).

Current data (DeHart *et al*., in review; this study) support the idea that serpentine endemics possess adaptations to elevated metal concentrations in serpentine soils (i.e., reduced uptake and translocation to leaves, reproductive organs and rewards) that non-endemics lack. These results suggest that non-endemic species may be at a fitness disadvantage compared to endemics when growing on serpentine soils. For example, in a series of experiments with *Mimulus guttatus* (Phrymaceae), Searcy and Mulcahy (1985) and Searcy and Macnair (1990) suggested that copper in the pistils of plants could act as a selective filter since seed production was reduced when pollen donors were not adapted to copper-rich soils. Floral metal accumulation may therefore produce a prezygotic isolating mechanism in non-endemic species compared to endemics by decreasing plant fitness when maternal and paternal plants are growing in different soil environments (i.e., serpentine and non-serpentine). In this way, floral metal accumulation may act as a reproductive barrier that favors reproduction between plants growing in similar soil environments, selecting against species that have serpentine and non-serpentine populations in close proximity to each other. Therefore, understanding metal accumulation into

flowers and floral rewards is vital not only for identifying potential reproductive costs associated with plant growth on metal-rich soils, such as serpentine, but also for explaining patterns of species distributions, reproductive isolation and plant endemism.

Metal accumulation by plants can be influenced by many factors, including phylogeny (Broadley *et al*., 2001) and maternal effects (Macnair 2002). Therefore, it must be acknowledged that many of the species used in this study, including all of the species in the endemic and indicator categories, belong to the same genus within the Brassicaceae, *Streptanthus*. However, the main comparisons of this study involved endemics vs. both indifferent and indicator species, with the latter group including members of several genera spread across multiple Tribes (*Boechera*: Boechereae; *Erysimum*: Erysimeae; *Hirschfeldia*: Brassiceae; *Streptanthus*: Schizopetaleae; Al-Shehbaz, 2013). Therefore, additional work comparing heavy metal accumulation across vegetative and reproductive organs of endemic and non-endemic plants from a variety of plant families (e.g., Asteraceae, Caryophyllaceae, Phrymaceae), and thus taking phylogenetic relationships into account regarding variation in metal accumulation, will contribute towards a more general understanding of edaphic endemism. Though not incorporated in the present study, the application of phylogenetically independent contrasts with paired endemic and non-endemic taxa (e.g., Cunningham *et al*., 1999) would be particularly informative when comparing Ni accumulation across levels of serpentine affinity. Furthermore, because seeds from each taxon were bulk-collected from a single population per species, intra-population level variation, if it exists (e.g., Macnair 2002), would be confounded with species. Our conclusions, however, are robust across affinity groups as each group includes two or more species. In addition, it is unlikely that maternal environmental effects influenced our findings as tissues were only collected from adult plants, and maternal affects generally

manifest in earlier life stages (i.e., seeds and seedlings) and decrease with plant age (Roach and Wulff 1987; Lopez et al. 2003; Donohue 2009). However, phytoavailable Ni concentrations in serpentine soils are well known to vary, both within and across regions containing serpentine soil (Echevarria *et al*., 2006). This variation can lead to ecotypic variation within species, with some populations being adapted to high phytoavailable Ni concentrations, while others are not (O'Dell and Rajakaruna 2011). Thus, future studies incorporating multiple populations will allow for further resolution of genetic and maternal environment effects on plant Ni accumulation, and whether this varies by serpentine affinity.

3.5 CONCLUSIONS

Although the current study does not assess differences in plant fitness or competitive ability between endemic and non-endemic plants, our results highlight consistent differences in heavy metal uptake between endemic and non-endemic serpentine species. While edaphic features of serpentine soils are known to influence plant fitness for non-endemic plants, both directly (e.g. Swope and Stein, 2012) and indirectly (e.g. Meindl *et al*., 2013), the specific effects of Ni accumulation on plant reproduction for non-hyperaccumulating serpentine species are not fully understood. Nickel tolerance has been identified as a key feature of serpentine soil tolerance (Alexander *et al*., 2007; Brady *et al*., 2005; O'Dell and Rajakaruna, 2011) and is generally accomplished through root sequestration or exclusion, though not all serpentine plant species effectively exclude Ni from above-ground tissues (reviewed in Alexander *et al*., 2007; O'Dell and Rajakaruna, 2011). Given the known toxicity of Ni for plant growth and reproduction (see

citations in Introduction), our study suggests that endemic and non-endemic plants may differ in reproductive potential (e.g. differences in seed production or pollinator visitation) when grown in serpentine soils due to differential Ni uptake and translocation. While our findings suggest that endemic species possess the ability to limit Ni uptake into above-ground tissues, future work assessing the fitness consequences of growth on serpentine soils will provide valuable information towards understanding edaphic endemism. However, studies like ours are necessary prerequisites for determining whether serpentine endemic and non-endemic species differ in reproductive or competitive capabilities (e.g. Imbert *et al*., 2011) due to differences in physiological response to soils.

Table 12. Species descriptions and seed collection locations for all plant species studied. Plants were divided into three categories: serpentine endemic, serpentine indicator, or serpentine indifferent. Serpentine affinity score is provided for all taxa, as defined by Safford et al. (2005)- species not discussed by Safford et al. (2005) are given a score of '<1'. Life history (annual or perennial) and distribution ranges are provided for all species (CA=California; NA=North America; AE=Afroeurasia).

Species	Plant Category	Habitat Affinity Score	Life History	Range	Seed Collection Location
S. breweri var. *breweri*	Endemic	5.7	Annual	CA	N 38°51'52.4"; W 122°24'16.4"
S. morrisonii	Endemic	6.1	Perennial	CA	N 38°48'45.3"; W 122°22'54.9"
S. glandulosus ssp. *glandulosus*	Indicator	1.9	Annual	CA	N 38°51'43.9"; W 122°23'57.3"
S. tortuosus	Indicator	1.7	Perennial	Western NA	N 39°59'18.4"; W 121°17'19.8"
Erysimum capitatum var. *capitatum*	Indifferent	<1	Perennial	NA	N 41°16'32.5"; W 122°41'54.4"
Hirschfeldia incana	Indifferent	<1	Annual	NA, AE	N 38°51'30.0"; W 122°24'35.2"
Boechera breweri	Indifferent	<1	Perennial	CA	N 39°57'12.3": W 121°19'4.5"

Table 13. Results from mixed model ANCOVA and pre-planned contrasts of Ni accumulation to leaves, pistils, anthers and nectar ('Organ/Reward Type') of seven mustard species that vary in their affinity to serpentine soil ('Habitat Affinity') when grown in either Ni-supplemented or control soils ('Soil Treatment'). The number of soil treatment applications ('Application Number') was included as a covariate. Random effects of individual plant ('Individual') and species (nested within habitat affinity; 'Species (Habitat Affinity)' were also included in the model. Significance of fixed effects denoted as *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.0001$.

Source of Variation	df (Num., Den.)	*F*
Habitat Affinity	2, 1.86	10.42
Soil Treatment	1, 119	1204.29***
Organ/Reward Type	3, 225	8.88***
Application Number	1, 13.3	26.57**
Habitat Affinity*Soil Treatment	2, 117	2.18
Habitat Affinity*Organ/Reward Type	6, 382	5.16***
Soil Treatment*Organ/Reward Type	3, 383	163.22***
Habitat Affinity*Soil Treatment*Organ/Reward Type	6, 383	2.24*
Random Effects		Z
Individual		3.28**
Species (Habitat Affinity)		0.19
Pre-planned Contrasts		
Endemic vs. Non-endemic (Indifferent/Indicator)		
Leaves:	1, 31.5	5.85*
Pistils:	1, 32.6	8.24**
Anthers:	1, 32.5	3.78
Nectar:	1, 32.5	0.02
Vegetative (Leaves) vs. Reproductive (Anthers/Pistils)		
Endemic:	1, 349	3.15
Indicator:	1, 321	0.01
Indifferent:	1, 384	20.42***

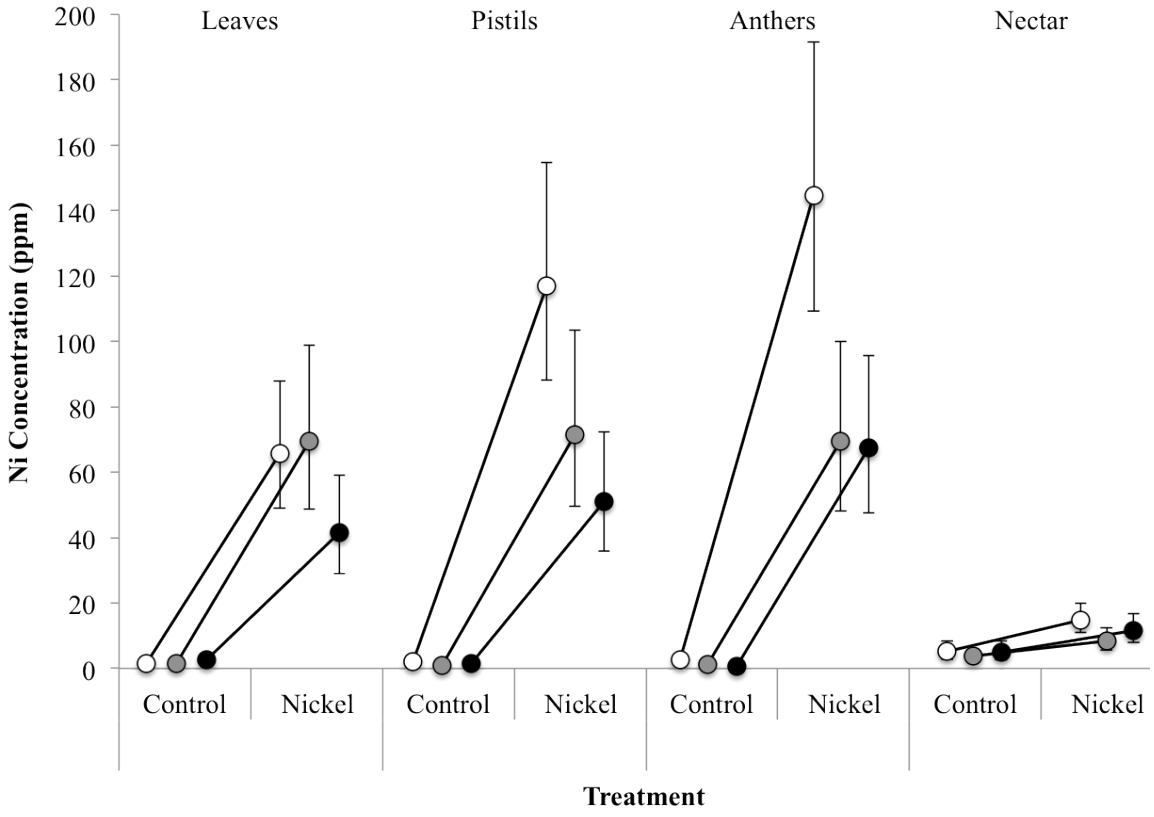

Figure 6. Nickel concentrations among serpentine indifferent (*Hirschfeldia incana*, *Erysimum capitatum* var. *capitatum*, *Boechera breweri*), indicator (*Streptanthus tortuosus, S. glandulosus* ssp. *glandulosus*) and endemic (*S. morrisonii, S. breweri* var. *breweri*) plant species when grown in control vs. nickel-supplemented soils by organ/reward type (vegetative organ [leaves], two reproductive organs [pistils, anthers] and one floral reward [nectar]). Symbols represent back-transformed lsmeans (± 95% CI). White symbols = indifferent species; gray symbols = indicator species; black symbols = endemic species.

4.0 VARIATION IN NICKEL ACCUMULATION IN LEAVES, REPRODUCTIVE ORGANS AND FLORAL REWARDS IN TWO HYPERACCUMULATING BRASSICACEAE SPECIES

4.1 INTRODUCTION

Metal hyperaccumulation refers to the excessive uptake and sequestration of soil metals by plants into above ground tissues and has been described in approximately 500 plant species (reviewed in van der Ent et al. 2013). For example, while most plants have <5 mg kg^{-1} nickel (Ni) in aboveground tissues (Marschner 2012), Ni hyperaccumulators exhibit tissue Ni concentrations >1,000 mg kg^{-1} (Reeves and Baker 2000). While several hypotheses have been proposed to explain the adaptive function of metal hyperaccumulation (e.g., elemental allelopathy, drought resistance and metal tolerance/disposal; reviewed in Boyd and Martens 1992), the hypothesis with the most experimental support is the 'elemental defense hypothesis' (Rascio and Navari-Izzo 2011). This hypothesis states that metal hyperaccumulation confers adaptive value to plants via protection from enemies, which has been shown in studies of both herbivores (Jhee et al. 2005) and pathogens (Boyd et al. 1994a). However, these benefits have only been considered for vegetative organs, despite the fact that flowering plants generally invest more defensive compounds in reproductive organs (e.g., Brown et al. 2003). With limited documentation of metal accumulation and hyperaccumulation into reproductive organs (Meindl

and Ashman 2014; Sánchez-Mata et al. 2014; Meindl et al. in review), it is unclear what fitness consequences may result from plants that exhibit the hyperaccumulation trait.

Floral metal hyperaccumulation is an important consideration, as metals hyperaccumulated in reproductive organs (including gametes) and rewards for pollinators (nectar, pollen) of plants may either have positive or negative effects on plant fitness. For example, metals hyperaccumulated in anthers and pistils could provide an elemental defense against herbivores and pathogens, similar to defenses provided to vegetative tissues (Boyd et al. 1994a,b; Jhee et al. 2005). Though metal hyperaccumulators have rarely been similarly studied (but see Meindl and Ashman 2014; Sánchez-Mata et al. 2014), elemental defense has been suggested for plants that hyperaccumulate the metalloid selenium (Se) into floral organs and rewards (Quinn et al. 2011; Prins et al. 2011). For example, Hladun et al. (2013) found that seed predation by birds was reduced for plants that accumulated high concentrations of Se. Conversely, metal hyperaccumulation may be limited to vegetative tissues so as to avoid interference with reproduction in gamete-producing organs. Furthermore, it is unknown whether hyperaccumulating plants generally accumulate metals into pollen grains or ovules (but see Sánchez-Mata et al. 2014). Pollen metal accumulation could influence plant fitness through reducing pollen germination (Mohsenzadeh et al. 2011; Yousefi et al. 2011a) or pollinator fitness (Moroń et al. 2012). Similarly, metals accumulated into pistils can reduce both ovule and seed viability for non-hyperaccumulating taxa (Malan and Farrant 1998; Yousefi et al 2011b). Documenting metal concentrations of reproductive organs and floral rewards of metal hyperaccumulating plants is a prerequisite for a holistic understanding of possible adaptive functions of metal hyperaccumulation.

Metals present in nectar can reduce pollinator foraging (Meindl and Ashman 2013; Meindl and Ashman 2014). Therefore, plant species that rely on pollinator visitation for sexual reproduction may limit floral metal accumulation, particularly in floral rewards (i.e., pollen and nectar), relative to species that rely less on biotic pollinators (e.g., those that are autonomously autogamous). While defensive secondary compounds in nectar, such as phenolics and alkaloids, have been hypothesized to benefit plant fitness, e.g., via increased outcrossing and/or decreased microbial degradation of nectar (Adler 2000), some data suggest that the costs of defensive compounds in nectar (e.g., reductions in pollinator visitation) may outweigh the benefits (Adler and Irwin 2005). Currently, however, there are no data available to compare floral metal accumulation across plant species that vary in their reliance on pollinators for reproduction.

Roughly 75% of metal hyperaccumulating plants are associated with the metal Ni, and approximately 25% of hyperaccumulating species belong to the Brassicaceae plant family (Reeves 2006; Rascio and Navari-Izzo 2011). In this study, we grew two species of Ni hyperaccumulating plants from the Brassicaceae family in either control soils or soils supplemented with Ni to determine whether Ni was concentrated into reproductive organs and floral rewards similarly to vegetative ones, and whether this varied between species as predicted by their reliance on pollinators for seed production.

4.2 MATERIALS AND METHODS

4.2.1 Study system

Two Ni hyperaccumulating species (*Streptanthus polygaloides* [yellow morph; Boyd et al. 2009]*), Noccaea fendleri* subsp. *glauca*) from the Brassicaceae family were used in this study. *Streptanthus polygaloides* is an annual species endemic to serpentine soils (> 95% of populations occur on serpentine soil; Safford et al. 2005) in northern California (Baldwin et al. 2012; Reeves et al. 1983). Hand pollinations show that *S. polygaloides* is partially self-compatible (Index of Self-Incompatibility (ISI) = 0.8, where ISI of 1 is considered self compatible, and ISI between 0.2 and 1 is considered partially self compatible (Zapata and Arroyo 1978); Meindl and Ashman, unpublished). It produces urceolate (i.e., urn-shaped) flowers that offer both pollen and nectar to pollinators. Pollinator visitation is required for sexual reproduction in this species (Boyd et al. 2009) and floral visitors are mainly bees, but also flies and beetles (Wall and Boyd 2002; Meindl and Ashman, unpublished). *Noccaea fendleri* subsp. *glauca* is a perennial species that is tolerant, but not strictly endemic, to serpentine soils (85-94% of populations occur on serpentine soil; Safford et al. 2005), and occurs throughout western North America (Al-Shehbaz 2012). *Noccaea fendleri* subsp. *glauca* is self-compatible (Meindl and Ashman, unpublished) and produces cruciferous flowers, typical of the Brassicaceae, which do not produce nectar. This species is highly autonomously autogamous in the greenhouse (Meindl, pers. obs.) and thus although bees and flies visit flowers in the wild pollinators are not required for seed set (Meindl and Ashman, unpublished). Both species are herbaceous and flower in the spring. Seeds from

each taxon were collected from the wild in the summer of 2012 (*S. polygaloides*: N 39°46'50.4", W 121°28'41.6"; *N. fendleri* subsp. *glauca*: N 41°16'42.4", W 122°41'48.7").

4.2.2 Experimental design

In the fall of 2012, twenty seeds per species (Total $N = 40$) were treated for two weeks with 4°C cold and dark conditions. Seedlings were transplanted to 27 cm^3 'rocket' pots (Deepots, Stuewe and Sons, Inc., Tangent, OR, USA) filled with standard potting soil (Fafard #4, Sun Gro Horticulture, Agawam, MA, USA) and six Nutricote® NPK 13-13-13 time-release fertilizer pellets (Arysta LifeScience Corporation, New York, NY, USA). One month after transplanting, *N. fendleri* subsp. *glauca* received a 4°C cold treatment for one month at 8D:16N. Subsequently, both *N. fendleri* subsp. *glauca* and *S. polygaloides* were grown under controlled conditions of 12D:12N and between 21.1-26.7°C until flowering in the greenhouse at the University of Pittsburgh.

One month after transplanting (*S. polygaloides*), or one week after cold treatment (*N. fendleri* subsp. *glauca*), plants were divided into two treatment groups ($N = 10$ plants / species / treatment) and soil treatment solutions were applied to each plant once per week: either (1) Ni-supplemented (40 mL of 400 mg kg^{-1} Ni nitrate ($Ni(NO_3)_2$-$6H_2O$) solution) or (2) control (40 mL of ammonium nitrate (NH_4NO_3) solution to control for 190 mg kg^{-1} nitrogen applied to plants in the Ni-supplemented treatment). Bioavailable fractions of Ni in serpentine soils generally range from 50 to 500 mg kg^{-1} (e.g., Echevarria et al. 2006; L'Huillier and Edighoffer 1996), thus our soil treatment solutions reflect a natural level of bioavailable Ni (resulting Ni concentration of

soil, by volume, was 592 mg kg^{-1} at time of watering). Soil treatments were conducted for each individual until flowering (4-14 weeks).

4.2.3 Organ/reward collection and chemical analysis

Three organs (leaves, pistils, anthers) and two floral rewards (pollen, nectar) were collected (nectar was not collected from *N. fendleri* subsp. *glauca* as it does not produce any). One fully developed leaf was collected from each individual following four soil treatment applications. Pistils, anthers, and nectar were collected from the first 5-15 flowers produced per plant, while pollen was collected from at least 10 flowers from two individuals per species-soil treatment from an independent set of mature anthers. For *S. polygaloides*, we collected nectar from all individuals by folding a circular piece of filter paper (Whatman® Grade 1, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) in half and touching it to the floral nectaries of several flowers per individual. We used Baker's (1979) spot-staining method, as described in Kearns and Inouye (1993), to determine nectar volume (see Meindl and Ashman 2014 for details).

Leaves and pistils were rinsed with diH_2O and dried at 60°C for 48 hours prior to chemical analysis. Anther, pollen, and nectar samples were allowed to air dry in microcentrifuge tubes for 48 hours. All samples were weighed to the nearest 0.0001 g and then microwave digested in 2-4 mL of trace metal grade HNO_3 and brought to a final volume of 12-14 mL with MilliQ (Millipore, Bedford, MA, USA) H_2O. Nickel concentrations are reported as mg kg^{-1} (organs and pollen) or uL L^{-1} (nectar) and were determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS, NEXION 300X, PerkinElmer, Waltham, MA, USA) at the University of Pittsburgh. A series of five samples, each with known Ni concentrations, were

used to construct standard calibration curves before running samples on the ICP-MS. Duplicate samples and blanks that contained internal standards were analyzed at regular intervals as measures of quality control during sample processing. All duplicate samples processed were within 10% of each other.

4.2.4 Statistical analysis

All statistical analyses were conducted in SAS (version 9.3; SAS Institute Inc., Cary, NC, USA). To evaluate the effect of Ni soil treatment, species, and organ type on plant Ni concentration, mixed-model ANCOVA was conducted (PROC MIXED). The model included the fixed effects of soil treatment, species, organ type (leaves, pistils, or anthers), and their interactions, and the random factor of individual. The number of soil treatment applications was included as a covariate. We used pre-planned contrasts to determine whether each hyperaccumulator species had similar Ni concentrations in reproductive organs (anthers, pistils) relative to leaves for plants in the Ni soil treatment only using the CONTRAST option (SAS 2011; Arceo-Gómez and Ashman 2014). Additionally, ANCOVA was used to compare Ni accumulation in nectar between control and Ni-treated *S. polygaloides*, with soil treatment as a fixed effect, individual as a random factor, and the number of soil treatment applications as the covariate. Denominator degrees of freedom for all *F*-tests were determined using the Kenward-Roger approximation (Littell et al. 2002). We used a student's *t*-test (PROC TTEST) to determine whether pollen Ni concentration was greater in Ni-treated plants than controls. Nickel concentrations were natural-log transformed to improve normality of residuals. Least squares means, which account for model covariates, (and 95% confidence intervals) were back-transformed for presentation.

4.3 RESULTS

Mean Ni concentrations in Ni-treated plants were more than 180 times higher than controls (Soil Treatment: $P < 0.0001$; Table 14; Fig. 1), and the effect of soil treatment varied by organ type (Soil Treatment x Organ Type: $P < 0.0001$; Table 14). In Ni-treated plants, Ni concentration was lowest in anthers (633 mg kg^{-1}), followed by pistils (758 mg kg^{-1}) and highest in leaves (1,200 mg kg^{-1}), whereas in control plants mean Ni concentrations ranged from 3-8 mg kg^{-1} across all organs (Fig. 1). While *N. fendleri* subsp. *glauca* accumulated twice as much Ni across both treatments relative to *S. polygaloides* (Species: $P < 0.0001$; Table 14; Fig. 1), the magnitude of this species difference varied by organ type (Species x Organ Type: $P < 0.0001$; Table 14). Furthermore, while Ni accumulation in leaves of Ni-treated plants was only 29% higher in *N. fendleri* subsp. *glauca* relative to *S. polygaloides*, *N. fendleri* subsp. *glauca* accumulated four times more Ni into pistils and three times more Ni into anthers (Table 14; Fig. 1). However, when averaged across all tissues Ni concentration was higher in Ni-treated plants than controls for both species (Species x Soil Treatment: $P > 0.05$; Table 14). Nickel accumulation varied significantly across individuals (Individual: $P < 0.05$; Table 14) but not by application number (Application Number: $P > 0.05$; Table 14).

When considering Ni-treated plants alone, however, organ-specific Ni accumulation varied between species (Species x Soil Treatment x Organ Type: $P < 0.01$; Table 14). Preplanned contrasts showed that within the Ni soil treatment, *S. polygaloides* had 64% lower Ni concentrations in both anthers and pistils relative to leaves (361 [95% CI: 247, 533] mg kg^{-1} vs. 1,002 (95% CI: 652, 1572) mg kg^{-1}; Table 14). Conversely, *N. fendleri* subsp. *glauca*

accumulated equal concentrations of Ni in both anthers and pistils relative to leaves (1,312 [95% CI: 934, 1863] mg kg^{-1} vs. 1,394 [95% CI: 907, 2186] mg kg^{-1}; Table 14).

Nickel-treated *S. polygaloides* plants accumulated ten times more Ni into nectar relative to control plants (Ni-treated: 61 mg kg^{-1}; control: 6 mg kg^{-1}; $F_{1,17} = 23.63$, $P < 0.0001$), but Ni accumulation was lowest in nectar relative to all other organs/rewards in this species (Fig. 1). Neither individual ($Z = 1.52$, $P > 0.05$) nor application number ($F_{1,17} = 23.63$, $P > 0.05$) influenced Ni accumulation in nectar by *S. polygaloides*.

Considering both species, mean Ni concentrations in pollen were 100 times higher in Ni-treated than control plants (229 mg kg^{-1} vs. 2 mg kg^{-1}; $t = -2.97$; df = 6; $P = 0.025$). While low sample sizes prevented a rigorous statistical comparison between species, Ni concentration of pollen from Ni-treated plants was higher for *N. fendleri* subsp. *glauca* (350 [95% CI: 54, 2242] mg kg^{-1}) than *S. polygaloides* (150 [95% CI: 23, 958] mg kg^{-1}).

4.4 DISCUSSION

Our study joins other recent work documenting Ni concentrations in floral organs and rewards for species that hyperaccumulate Ni (Meindl and Ashman 2014; Sánchez-Mata et al. 2014). However, our work is novel by extending these findings to include two species that vary in mating system and reliance on biotic pollination. Both Ni hyperaccumulating species studied here concentrated Ni into reproductive organs, though *N. fendleri* subsp. *glauca* had similar concentrations of Ni across all organs while *S. polygaloides* accumulated less Ni into anthers and

pistils relative to leaves. *Streptanthus polygaloides* incorporated Ni into nectar, and both species incorporated Ni into pollen.

While ours is among the first to experimentally determine Ni concentrations in reproductive organs of Ni-hyperaccumulators, several studies of selenium (Se) hyperaccumulators have found similar results (Prins et al. 2011; Quinn et al. 2011; Valdez Barillas et al. 2012). For example, Quinn et al. (2011) discovered that the Se-hyperaccumulator *Stanleya pinnata* (Brassicaceae) hyperaccumulated Se in flowers to the same degree as leaves, and that the highest Se concentrations in flowers were found in anthers, pistils and young seeds, though high concentrations of Se were also detected in pollen and ovules. Interestingly, studies have found no evidence for fitness costs of Se hyperaccumulation, as pollen germination and pollinator visitation are not reduced by Se hyperaccumulation (Quinn et al. 2011). In fact, pollen germination has been observed to be higher for Se hyperaccumulating plants supplied with elevated levels of Se in soils (Prins et al. 2011). For the heavy metal Ni, specifically, studies have shown that Ni can be hyperaccumulated into seeds and that seed Ni concentration is positively correlated with shoot Ni concentration (Brooks 1998; Psaras and Manetas 2001; Adamidis et al. 2014). While the fitness consequences of Ni hyperaccumulation in seeds have not been similarly studied, hyperaccumulation of another heavy metal (cadmium) in seeds is known to reduce seed viability in hyperaccumulating species, indicating a fitness cost to floral metal hyperaccumulation (Vogel-Mikuš et al. 2007). Here we show that Ni is accumulated in high concentrations in floral organs of two Ni hyperaccumulating species, but it is unclear at this time what effects this may have on pollen, ovule or seed viability in these species.

Concentrations of Ni in leaves relative to reproductive organs and rewards varied across the two species studied under controlled conditions (Fig. 1), and corroborate differences seen in

organs collected from field-grown plants. Specifically, anther Ni concentrations in field grown plants of *N. fendleri* subsp. *glauca* can average above 3,000 mg kg^{-1}, whereas those of *S. polygaloides* average only ~ 2,000 mg kg^{-1} (Meindl and Ashman, unpublished). Because both species are known to hyperaccumulate Ni into reproductive organs in natural populations, a lack of hyperaccumulation observed in some organs of *S. polygaloides* in this study may reflect a comparatively inconstant supply of Ni in the greenhouse environment relative to natural soil conditions. However, regardless of whether the two species reached hyperaccumulation thresholds in our greenhouse experiment, observed differences between species in floral Ni accumulation suggests that some species may experience reproductive costs of floral metal accumulation, whereas others may not. There are three possible explanations for the differences in Ni accumulation in reproductive organs observed between species. First, previous work has shown that serpentine affinity, i.e., whether plants are endemic or not, can influence Ni accumulation into both leaves and flowers with endemic taxa generally accumulating lower levels of Ni into organs (DeHart et al. in review; Meindl et al. in review). However, this is unlikely to account for the differences seen in this study, as these species are both very closely associated with serpentine. Indeed, Safford et al. (2005) refer to *N. fendleri* subsp. *glauca* (a.k.a., *Thlaspi montanum* var. *montanum*) as a 'broad endemic' because it is most often associated with serpentine soils, despite not being entirely restricted there. However, additional work that replicates endemic and non-endemic species of metal hyperaccumulators would be required to determine definitively whether serpentine affinity alters metal hyperaccumulation. Second, because excessive accumulation of Ni into floral rewards may be detrimental to plant fitness (Meindl and Ashman 2013; Meindl and Ashman 2014) the lower relative concentration of Ni in nectar of *S. polygaloides* and the lower concentration of Ni in its pollen relative to *N. fendleri*

subsp. *glauca* may be explained by the fact that *S. polygaloides* is only partially self-compatible and relies on pollinating insects for seed and fruit production (Boyd et al. 2009). This contrasts with *N. fendleri* subsp. *glauca*, which is self-compatible and autonomously autogamous (Meindl and Ashman, unpublished), so the lower dependence on insect pollinators for reproduction may mean that if floral metal accumulation reduces pollinator visitation it will not have as great a fitness cost as it might for *S. polygaloides*. Third, species may vary in their reliance on floral metal accumulation for elemental defense of reproductive organs. However, the costs vs. benefits of floral metal hyperaccumulation require additional study to determine whether it serves an adaptive function, or simply reflects inadvertent uptake (e.g., Boyd and Martens 1992).

Since the fitness consequences of floral Ni hyperaccumulation for these species are not yet known we cannot conclude whether it is adaptive or maladaptive. This will require determining the effects of metal hyperaccumulation on plant fitness at each reproductive stage (i.e., flower production through seed germination). For example, hyperaccumulation may have fitness advantages such as increased flowering (Ghasemi et al. 2014), pollen germination (Prins et al. 2011), or seed germination (e.g., the Ni hyperaccumulator *Alyssum murale* (Brassicaceae); M. McKenna, pers. comm.). However, trade offs may exist at other stages of reproduction (e.g., reducing pollinator visitation) so all stages need to be considered. Taken together, evidence is mounting that Ni and other metals are concentrated in reproductive organs and rewards at high levels, thus the next step for research in these systems is determining fitness consequences. Such work will provide a complete evaluation of the adaptive potential and ecological consequences of plant hyperaccumulation.

Table 14. Results from mixed-model ANCOVA and pre-planned contrasts of Ni concentrations in leaves, pistils and anthers ('Organ Type') of two Ni-hyperaccumulating species (*Streptanthus polygaloides* and *Noccaea fendleri* subsp. *glauca*; 'Species') when grown in either Ni-supplemented or control soils ('Soil Treatment'). The number of treatments applied to soils ('Application Number') was included as a covariate. Significance of fixed effects denoted as **P*≤0.05, ***P*≤0.01 and ****P*≤0.0001.

Source of Variation	**df (Num., Den.)**	***F***
Species	1, 43.5	24.85***
Soil Treatment	1, 35.4	1181.83***
Organ Type	2, 80.8	2.5
Application Number	1, 99	1.01
Species*Soil Treatment	1, 35.4	0.82
Species*Organ Type	2, 78.2	29.03***
Soil Treatment*Organ Type	2, 71.3	24.68***
Species*Soil Treatment*Organ Type	2, 71.3	7.73**
Random Effect		*Z*
Individual		2.27*
Pre-planned Contrasts for Ni-treatment		
Leaves vs. Anthers/Pistils		
Streptanthus polygaloides:	1, 89.7	17.26***
Noccaea fendleri subsp. *glauca*:	1, 71.7	0.10

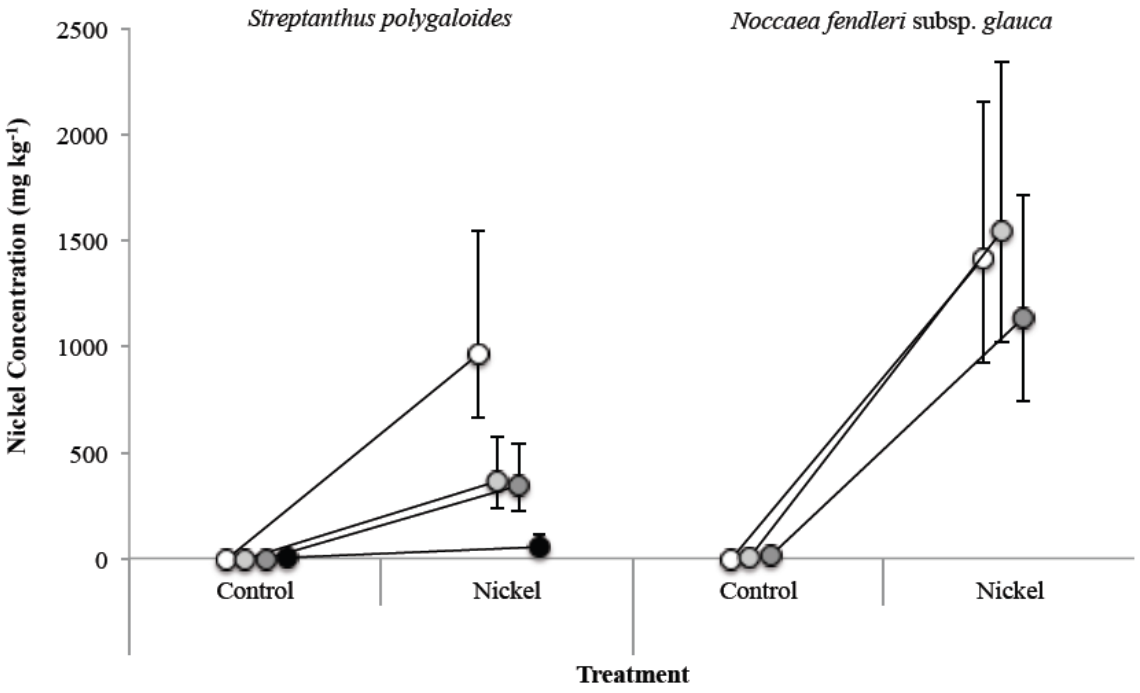

Figure 7. Differences in Ni concentration (mg kg^{-1}) between two Ni-hyperaccumulating species (*Streptanthus polygaloides* and *Noccaea fendleri* subsp. *glauca* grown in control vs. Ni-supplemented soils across one vegetative organ (leaves), two reproductive organs (pistils, anthers) and one floral reward (nectar). Nectar was produced by *S. polygaloides* only. Symbols represent back-transformed lsmeans (± 95% CI). White symbols = leaves; light gray symbols = pistils; dark gray symbols = anthers; black symbols = nectar.

5.0 THE EFFECTS OF ALUMINUM AND NICKEL IN NECTAR ON THE FORAGING BEHAVIOR OF BUMBLEBEES

5.1 INTRODUCTION

Heavy metals occur in small amounts naturally in most soils, but anthropogenic activities (e.g., industry, agriculture) can lead to toxic levels (Nagajyoti et al. 2010). Specifically, nickel (Ni) and aluminum (Al) are among the most common soil metal pollutants (e.g., Abollino et al. 2002) and they are considered toxic at high concentrations to both plants and animals (Sparling and Lowe 1996; Nagajyoti et al. 2010; Boyd 2010). Plants that accumulate these metals into vegetative tissues may be well defended against herbivory (but see Boyd 2009), as metals can decrease the growth and survival of insects (e.g., Boyd and Martens 1994; Boyd 2010). It is unclear, however, whether metals present in flowers alter how pollinators interact with plants growing in contaminated soils, and whether pollinators might be subjected to potentially toxic effects.

As metals and metalloids can be transferred to pollen grains (Se: Quinn et al. 2011) and nectar (Se: Hladun et al. 2011; Ni: Meindl and Ashman, unpublished data), Ni and Al contamination may negatively impact plant reproduction, including pollen-pistil interactions (Searcy and Macnair 1985) and plant-pollinator interactions. Ni and Al have been shown to decrease growth and survival of various insects (Boyd and Martens 1994; Sparling and Lowe

1996); therefore it is possible that bees collecting contaminated floral rewards will also experience toxic effects (e.g., Hladun et al. 2012). Although many plants exclude metals, some tolerant plants are known to accumulate metals into above ground tissues at high concentrations (e.g., metal hyperaccumulators; van der Ent et al. 2013). While an understanding of how soil pollutants affect plant-pollinator interactions is generally important, this issue is particularly relevant when assessing the potential impacts of phytoremediation with insect-pollinated hyperaccumulating plants, some of which are grown through flowering in the field (Chaney et al. 2010).

The potential for metals to be transferred from soils to higher trophic levels, and especially to affect pollinators, is largely unknown (but see Boyd 2009 for review of Ni transfer to insects). For example, while bumblebees avoid and/or spend less time foraging on flowers with relatively low rewards (e.g., poor pollen quality; Robertson et al. 1999), we currently do not know whether bumblebees will avoid or forage indiscriminately on metal-tainted flowers (but see Quinn et al. 2011 and Hladun et al. 2013 for effects of Se on visitation). Bumblebees, which are important pollinators both in agricultural and natural settings, are declining in many areas (Goulson 2009), thus understanding how soil pollution may alter foraging will be important for managing both plants and bumblebees in polluted landscapes. As a first step, it is critical to understand how bumblebees respond to plants that contain metal-tainted floral rewards. To address this gap in our understanding, here we answer the following questions: (1) Are flowers with metal-tainted nectar less likely to be visited by bumblebees compared to flowers with metal-free nectar? (2) Do bumblebees spend less time foraging on flowers with metal-tainted nectar? (3) Do bumblebees that sample flowers with metal-tainted nectar subsequently discriminate against nearby flowers?

5.2 METHODS

5.2.1 Study system

Impatiens capensis Meerb. (Balsaminaceae) is an herbaceous annual species native to North America that blooms throughout the summer (May-September) in western Pennsylvania (Rhoads and Block 2007). Its flowers are large and produce ~5 μL of 40% sucrose nectar per day (Rust 1977). Populations of *I. capensis* growing on metal contaminated soils have been shown to accumulate several metals, including Ni, into tissues at concentrations approaching those defining hyperaccumulators (e.g., ~800 ppm Ni; Curran 2005).

5.2.2 Study Site

This study was conducted at Carnegie Museum of Natural History's Powdermill Nature Reserve in Westmoreland Co., PA, USA (40°10'N, 79°16'W) between August 24 and September 1, 2012. *Impatiens capensis* is a common understory flowering plant throughout the reserve, found along creeks, wet ditches and roadsides. *Impatiens capensis* at the reserve had tissue concentrations of Al and Ni (mean (±SE): leaves 22.6 (±1.9) ppm Al, 3.8 (±0.2) ppm Ni; flowers 36.0 (±5.0) ppm Al, 3.3 (±0.3) ppm Ni; N=5 per tissue; unpublished data) similar to other temperate herbaceous plants (e.g., McGee et al. 2007; Metali et al. 2012). Mean total Al and Ni concentrations in soils near the study site were 5,624.6 ppm and 6.3 ppm, respectively (N=3; unpublished data), which are typical for the continental U.S., though Al concentration is lower than previously reported for the region (Shacklette and Boerngen 1984). Plants were visited

primarily by one species of bumblebee (*Bombus impatiens*). In addition to studying the effects of Ni in nectar, we also chose Al as some areas on the reserve are historically known to be contaminated with this metal (Mulvihill et al. 2008). Both Ni (van der Ent et al. 2013) and Al (Metali et al. 2012) are hyperaccumulated by several plant species.

5.2.3 Experimental Design

To determine whether Ni and Al in nectar individually influence bumblebee behavior, we created arrays of field-collected flowers. Flowers were collected in the morning, and placed in 225 mL centrifuge tubes filled with water and topped with florist's foam. Each individual flower was collected from a separate plant within a single population, and all flowers selected were of similar age, color and size. Using a micropipette, the standing nectar was removed from each flower, and replaced with 5 μL of an artificial nectar solution. One of three treatments was applied randomly to each flower: (1) 40% sucrose solution (control), (2) 40% sucrose solution with 100 ppm Ni, or (3) 40% sucrose solution with 100 ppm Al. These metal concentrations were chosen as previous work indicates that one Ni hyperaccumulating species accumulates at least 100 ppm Ni into nectar when grown in high-Ni soils (*Streptanthus polygaloides*; Meindl and Ashman, unpublished data). Both Ni and Al solutions were prepared using metal nitrates ($Al(NO_2)_3$ and $Ni(NO_2)_3$).

Each array consisted of four flowers: two control and two metal-treated flowers (Figure 8). Ni and Al were tested against control in separate experiments. A pair of each type of flowers were placed approximately four cm from one another and 20 cm from the other pair. When a bumblebee visited a flower in an array, the entire visitation sequence was recorded, as well as the

time spent foraging (in seconds) on each individual flower. Following an observed visitation sequence, all flowers in the array were replaced with unvisited flowers, and the position of the control and metal-treated flowers in the array was switched to avoid spatial bias. Only bumblebee nectaring bouts are considered here; if flowers were visited by non-bumblebee pollinators, or were visited by bumblebees foraging for pollen, flowers were not scored and replaced with unvisited flowers. On most days of observation, arrays composed of both types (with Al or Ni) were observed, with metal type alternating between early and late in the day (total N=145 arrays). Flower width was measured for each experimental flower for use as a covariate in analysis of bumblebee foraging time.

All statistical tests were performed using SAS Version 9.3 (SAS 2010). To determine whether the presence of metals in nectar affected the probability of a flower being visited, we used chi-square analysis (PROC FREQ) where the null hypothesis was that control and metal-treated flowers were equally likely to be visited. The effect of metal-treated nectar on time spent foraging by bumblebees was determined using ANCOVA (PROC GLM). Independent variables included date, time of day and flower width (covariate). Time spent foraging was log transformed to meet the assumption of normality. Logistic regression (PROC GENMOD) was used to determine if a bumblebee's subsequent decision was influenced by whether it first visited a metal-treated or a control flower. Bumblebee decisions following the initial visit were categorized as: (1) moved to the flower immediately adjacent, i.e., same treatment, (2) moved to a flower in the alternate treatment, or (3) the bumblebee left the array without visiting other flowers within it. The factors in the model included the bumblebee's first choice (control or metal-treated flower), date and time of day. In all models, Ni and Al arrays were analyzed separately.

5.3 RESULTS AND DISCUSSION

Metal-tainted nectar altered bumblebee visitation, but the extent of the effect depended on both the response variable and the metal type. The probability of a flower being visited did not depend on the presence of metals in nectar for either metal (Al: χ^2=0.07, df=1, P=0.4; Ni: χ^2=2.41, df=1, P=0.06). However, the time a bee spent foraging was affected by Ni, but not Al, in the nectar. Bumblebees spent 75% less time foraging on flowers containing Ni relative to controls ($F_{1,175}$=102.96; P<0.0001), while there was no difference between foraging time on Al and control flowers ($F_{1,137}$=1.07; P=0.15; Figure 9). Flower width did not influence visitation rate for flowers in either Ni ($F_{1,175}$=2.39; P=0.12) or Al ($F_{1,137}$=3.86; P=0.06) arrays. Nickel in nectar influenced the next foraging decision by a bumblebee (χ^2=3.37, df=1, P=0.03), whereas Al in nectar had no such effect (χ^2=0.00, df=1, P=0.48). Specifically, bumblebees that visited control flowers first were more likely to visit the next closest flower (i.e., same treatment) in the array than those that first visited Ni-treated flowers (49% vs. 35%). In addition, bumblebees that visited control flowers first were less likely to leave the array entirely without visiting any other flowers than bees that first visited Ni-treated flowers (16% vs. 33%; Figure 10).

Our work shows that metals present in nectar can alter the way pollinators interact with plants. Since flowers with control or metal-treated nectar were equally likely to be visited by bumblebees we conclude that bees do not detect metals in nectar from afar, and thus do not initially avoid flowers with metal-tainted nectar. However, once bumblebees arrive at flowers and sample the nectar, they are able to discriminate against certain metals, in this case Ni. Nickel in vegetative tissues has been shown to deter herbivores (Strauss and Boyd 2011), and here we show that Ni in nectar can reduce visitation by bumblebees. Aluminum, however,

produced no such effect, as bees foraged on Al-treated flowers for periods of time equal to that of bees foraging on controls. It is unclear why bees were not deterred by the presence of Al in nectar, however studies of honeybee chemistry have shown Al concentrations in bees to be much higher than Ni concentrations (van der Steen and de Kraker 2012), suggesting bees may be more tolerant to Al-tainted resources. Our findings suggest that metal pollution will have element-specific effects on the behavior of local pollinators.

The potential for soil metals to cascade through plants to affect pollinators is understudied, yet as humans continue to modify natural habitats understanding the transfer of soil contaminants to higher trophic levels will be vital for preserving ecosystem health and function. Results from our study may be particularly relevant when considering the use of insect-pollinated plants for the purpose of phytoremediation. Phytoremediation generally involves the use of metal hyperaccumulating plants to remove heavy metal soil contaminants from polluted soils (Chaney et al. 2010). Despite the apparent benefits of phytoremediation, land managers have not fully considered how interactions with insect herbivores and pollinators may be affected, especially considering that some hyperaccumulating plants are bumblebee pollinated (Quinn et al. 2011). While bumblebees did not spend as much time foraging on flowers with Ni-tainted nectar, they still visited these flowers, and therefore likely ingested a potentially toxic resource. Because metals can be transferred from hyperaccumulating plants to insect pollinators, phytoremediation with insect-pollinated flowering plants should be considered with caution (Wu et al. 2010).

Bioaccumulation of heavy metals has been observed in several insect food webs (e.g., Peterson et al. 2003). Predatory insects and arachnids have been documented to accumulate heavy metals from their insect prey near mining and smelting operations (Hunter et al. 1987;

Nummelin et al. 2007). If bioaccumulation of heavy metals is occurring similarly in plant-pollinator systems then it could threaten native pollinator populations, which are valuable both in terms of wild ecosystem services as well as in agricultural settings where reliance on honey bee colonies is threatened (Potts et al. 2010). Recent studies highlight the negative effects Al and Ni can have on insect physiology- Al exposure can cause severe neurological damage in flies, causing defects in locomotion and learning ability (Wu et al. 2012), while Ni can lower insect immune response (Sun et al. 2011). In addition, transfer of metals to bees may also impact human health, as toxic metals have been detected in honey samples collected near polluted environments (e.g., Citak et al. 2012). Therefore, understanding the ultimate fate of soil metal contaminants is critical for the health of both plants and animals.

Figure 8. Example of experimental array. Arrays consisted of four field-collected flowers placed in water-filled centrifuge tubes. Two metal-treated flowers were placed 4 cm apart, and 20 cm apart from a pair of control flowers. Following an observed visitation sequence, all flowers in an array were replaced with unvisited flowers, and the location of metal-treated and control flowers were switched.

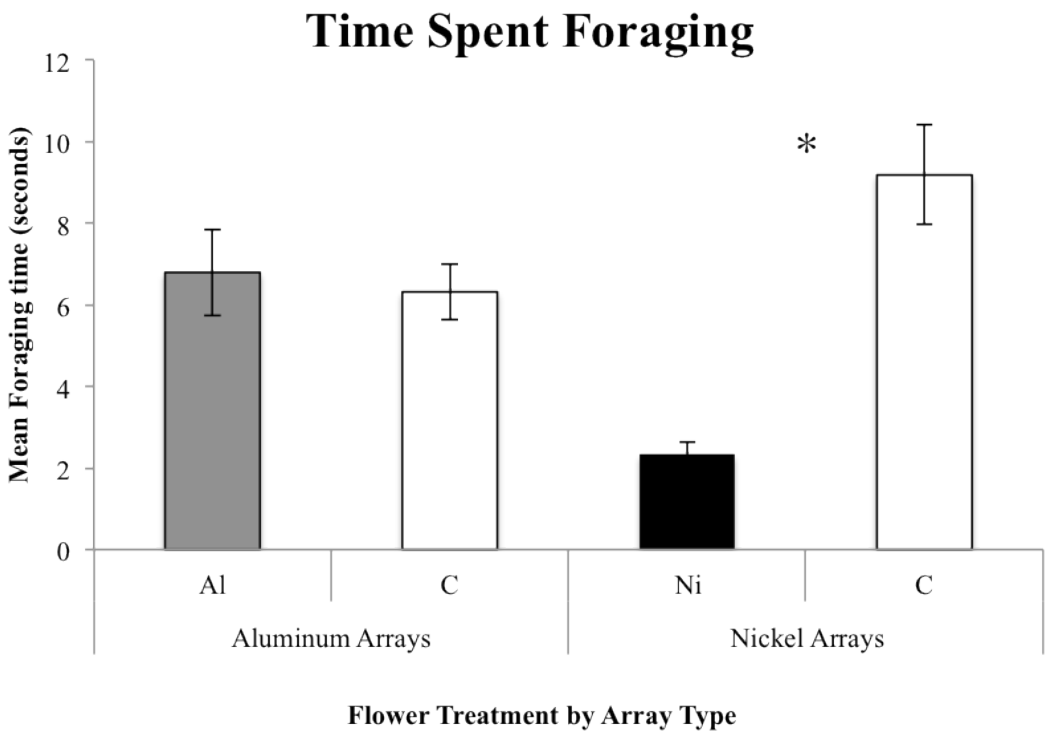

Figure 9. Mean foraging time (± SE) by bumblebees to treatment flowers. Asterisks indicate significant differences between treatments (*P*<0.05).

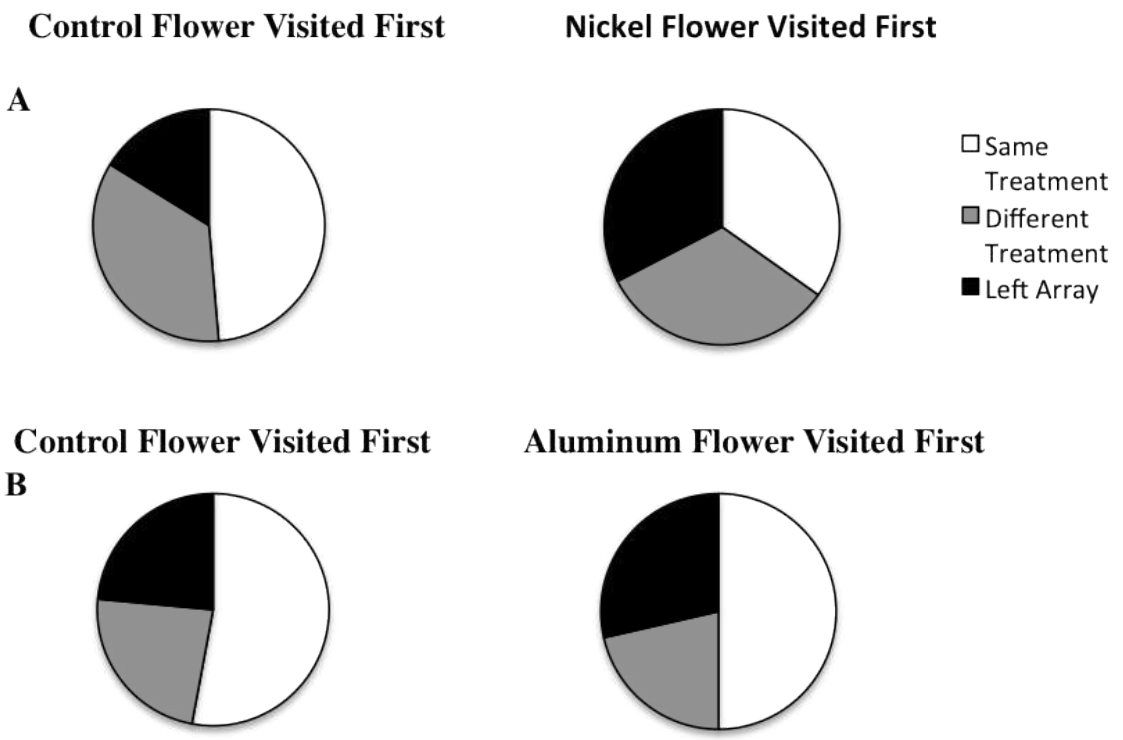

Figure 10. The proportion of bumblebees that either visited the next closest flower (of the same treatment; white section), one of the two flowers in the other treatment in the array (grey section), or left the array entirely (black section). Data presented for both (A) Ni arrays and (B) Al arrays.

6.0 NICKEL ACCUMULATION BY *STREPTANTHUS POLYGALOIDES* (BRASSICACEAE) REDUCES FLORAL VISITATION RATE

6.1 INTRODUCTION

Metal hyperaccumulation is a phenomenon described in over 500 plant species, representing 101 families (Sarma 2011), and refers to the uptake and sequestration of soil metals, e.g., copper, nickel, and zinc, into above ground tissues (reviewed in van der Ent et al. 2013). Concentrations that define hyperaccumulators vary by metal, although thresholds are generally at least one order of magnitude higher than average metal concentrations in plant tissues (e.g., Nickel [Ni] hyperaccumulators have at least 1,000 ppm Ni dry weight in tissues, while average Ni concentrations in plant tissues are <10 ppm; van der Ent et al. 2013). Roughly three quarters of metal hyperaccumulating plants are accumulators of the metal Ni and occur on serpentine soils (Reeves 2006), which are derived from metal-rich ultramafic rocks (Alexander et al. 2007). Despite the documented abundance of natural populations of Ni hyperaccumulating plants, the adaptive value and ecological significance of Ni hyperaccumulation for plants is still uncertain (Boyd and Martens 1992; Boyd 2004).

Several hypotheses regarding the adaptive value of metal hyperaccumulation have been proposed, including its role in interference (i.e., elemental allelopathy), drought resistance, and defense against antagonists (reviewed in Boyd and Martens 1992). However, special attention

has been paid to plant-herbivore interactions. Specifically, Boyd and Martens (1992) posit that metal hyperaccumulation in vegetative tissues provides defense against insect herbivores and bacterial/fungal pathogens, as moderate to high concentrations of metals in tissues can be toxic to many organisms (Coleman et al. 2005). Metals in vegetative tissues can act as feeding deterrents (Boyd and Jhee 2005), or, when ingested, can decrease growth and survival of insect herbivores (Boyd and Martens 1994). Beyond impacts on herbivory, however, little is known about how metal hyperaccumulation in plant tissues may alter ecological interactions, particularly mutualistic interactions. Of the limited studies in terrestrial systems, evidence suggests prolonged metal exposure has led to divergent microbial communities in the guts of soil-dwelling isopods (Lapanje et al. 2010) and decreased mycorrhizal abundance and diversity on plants growing in metal-polluted environments (Leyval et al. 1997; Vogel-Mikus et al. 2005). However, effects of metal accumulation in flowers on plant-pollinator interactions remain largely unstudied, despite the fact that toxic plant secondary chemicals, e.g., alkaloids, in nectar are well known to alter pollinator foraging (reviewed in Adler 2001).

Metals are known to accumulate in flowers of metal hyperaccumulators (Baker and Brooks 1989), but floral rewards (i.e., nectar and pollen) have rarely been separately evaluated. Recent experiments have shown that Ni in artificial nectar solutions decreases visitation by bumblebees (Meindl and Ashman 2013). In contrast, recent studies with selenium (Se) hyperaccumulator and non-hyperaccumulator taxa suggest that Se accumulation does not influence pollinator visitation (Hladun et al. 2013; Quinn et al. 2011). Considering that Se is toxic to honeybees (Hladun et al. 2012), generalist naïve pollinators (i.e., those not adapted to Se-rich floral rewards) foraging on Se-rich flowers may suffer negative fitness consequences. Similar effects may be seen for metal hyperaccumulating plants, since some bees cannot detect

Ni in flowers prior to visitation (Meindl and Ashman 2013) although Ni is toxic to insect herbivores (Boyd and Jhee 2005). Our study was designed to determine whether (1) Ni hyperaccumulating plants accumulate Ni into floral rewards (e.g., nectar and pollen) and (2) generalist floral visitors forage indiscriminately on Ni-rich flowers. These determinations will inform pollination ecology of metal hyperaccumulating plants in natural populations, as well as provide insight into ecological consequences of using these plants to remediate metal contaminated soils (i.e., phytoremediation; Pilon-Smits 2005).

While metals present in flowers may influence pollinator visitation directly (e.g., Meindl and Ashman 2013), there may also be indirect effects via modification of floral traits important for pollinator attraction, such as flower production and floral reward quantity. For example, heavy metals such as copper and Ni can delay flowering (Brun et al. 2003) and decrease total production of flowers per plant (Saikkonen et al. 1998), which in turn can reduce pollinator visitation (Mitchell et al. 2004). Furthermore, soil metals can decrease viable pollen production/flower (Slomka et al. 2012) and thereby lower the quantity and quality of rewards offered to pollinators, though effects of metals on nectar production and chemistry are unknown. Thus, soil metals may alter pollinator visitation to plants growing in metal-rich soils either directly by altering reward amount (i.e., reward quantity) or chemical composition (i.e., reward quality), or indirectly by altering floral display.

Here, we provide an initial test of whether Ni accumulation by a serpentine-endemic Ni hyperaccumulator (*Streptanthus polygaloides* Gray [Brassicaceae]) alters floral display, floral reward quantity and quality, and visitation by naïve floral visitors. Specifically, we address the following questions: (1) Does short-term exposure to Ni-supplemented soil alter floral display or quantity of nectar and pollen? (2) Is soil Ni absorbed and incorporated into floral

nectar and pollen? (3) Does soil Ni alter the likelihood a plant is visited by flower-visiting insects or its overall visitation rate per flower?

6.2 METHODS AND MATERIALS

6.2.1 Study system

Streptanthus polygaloides is an annual serpentine endemic (Baldwin et al. 2012; Reeves et al. 1981) and a Ni hyperaccumulator (Reeves et al. 1981). Its zygomorphic flowers attract bees, flies and beetles (e.g., *Dianthidium* spp., *Ceratina* spp., *Apis mellifera* (Hymenoptera), Syrphidae (Diptera), Bupresitidae (Coleoptera); Wall and Boyd 2002; unpublished data) that feed on pollen from exerted anthers (Preston 1991) and nectar produced at the base of stamens. Individual flowers remain open for at least four days (unpublished data).

6.2.2 Experimental design

Seeds collected from a population (37°36′48.71″N, 120°08′22.08″W) in Mariposa County, CA, were germinated on a thin layer of perlite (~6 mm) in 27cm^3 'rocket' pots (Deepots, Stuewe and Sons, Inc.) filled with potting soil (Fafard® #4) in a greenhouse at the University of Pittsburgh. After twelve weeks of growth 48 plants were moved to a site in an open field at the Powdermill Nature Reserve in western PA (40°10'N, 79°16'W). This site provided abundant generalist pollinators (unpublished data). At onset of flowering during June 2012, plants were divided into two soil treatments: + Ni and control (no Ni added to soil). Nickel was applied to Ni-treated

plants by top-watering once per day with 40 mL of solution containing 200 ppm Ni for 14 days prior to floral visitor observation experiments. Because metal hyperaccumulating plants have a high affinity for metals and thus the ability to rapidly acquire them from soils (Li et al. 2003), we applied solution treatments during flowering to ensure that Ni was available to plants during flower production. While this may not simulate natural conditions, it allows us to focus on Ni accumulation rather than other ontogenetic changes that might occur over long periods of exposure. Nickel treatment solutions were prepared using Ni nitrate ($Ni(NO_3)_2$), a Ni salt commonly used for studies of Ni hyperaccumulation (e.g., Kramer et al. 1997). Treatment solution was slowly and carefully applied to the soil surface using a plastic syringe, such that solution did not excessively contact shoots. Serpentine soils contain phytoavailable fractions of Ni that generally range from 50 ppm to 500 ppm (Chardot et al. 2005; L'Huillier and Edighoffer 1996), thus the Ni concentration used here to treat soils is conservative. Nitrates do not directly affect pollinator visitation generally (Burkle and Irwin 2010), so it is assumed that any differences in visitation between treatments here are due to differences in Ni concentrations. Furthermore, the short duration of treatment application makes it unlikely that nitrates significantly altered floral visitation, as even long-term nitrogen supplementation experiments have failed to find strong effects of nitrogen addition on floral visitation (Burkle and Irwin 2010). Control plants were similarly top-watered with 40 mL of pure water for 14 days prior to floral visitor observation experiments. When plants were not being observed (see below), they were kept under an awning to protect them from occasional rainfall.

6.2.3 Floral measurements

Prior to pollinator observations, we measured flower size with digital calipers (the product of flower [i.e., perianth] length and width, in mm) for two randomly selected flowers per plant. We also counted the number of open flowers per plant. Open flowers and flower size were enumerated once at the beginning of each week of observation. Following floral visitor observations, plants were moved indoors and we collected anthers and nectar. For each individual plant, all six anthers were collected from ten mature but unopened buds (i.e., 60 anthers collected per plant), air dried for 48 hours, and then weighed on a AE200 Mettler® analytical balance to the nearest 0.0001 g. Nectar was allowed to accumulate within flowers for 12 hours, and then it was collected on filter paper wicks (Whatman®, Grade 1) from four flowers per plant (generally 3-4 μL collected per plant). Folding a circular piece of filter paper in half, and then touching the folded edge to the floral nectaries, we collected nectar consistently in a circular pattern. Nectar volume was determined via Baker's (1979) spot-staining method, as described in Kearns and Inouye (1993), by comparing the measured diameter (mm) of each circular nectar spot on filter paper to a table of nectar spot diameters corresponding to nectar volumes (μL). This technique is valid for nectars with sugar concentrations ranging from 10-50%, which is in the range of many Brassicaceae species (Masierowska 2003), and provided that nectar spot diameters are ≤12 mm, which was the case in our study. Average anther mass and nectar volume per flower were calculated as estimates of reward quantity. Anther and nectar samples were pooled (separately) within individual plants for chemical analysis; thus each individual plant was treated as a separate replicate providing one nectar and one anther sample. We assume that anther mass and number of pollen grains per anther are positively correlated, as

has been shown for other species (Bhowmik and Datta 2013), and hereafter consider anther mass as a measure of pollen quantity.

6.2.4 Floral reward analysis

Anther and nectar samples from each plant were microwave digested in two mL of trace metal grade HNO_3 and brought to a final volume of 12 mL with MilliQ H_2O (Millipore, Bedford, MA, USA). A five mL aliquot of diluted digest was further diluted with five mL of 2% HNO_3 solution and mixed with a small volume (80 μL) of known concentrations of three internal standards (Beryllium, Germanium, Thallium). Concentration of Ni in anthers (mg kg^{-1}) and nectar (μL L^{-1}) were determined via Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Perkin/Elmer NEXION 300X) at the University of Pittsburgh. A series of five samples with known Ni concentrations were used to construct standard calibration curves prior to each run of samples on the ICP-MS. Duplicate samples and blanks, each containing internal standards, were analyzed at regular intervals as a measure of quality control during sample processing on the ICP-MS, and were within 10% of each other. Control filter paper wicks were also processed to verify the absence of Ni in the filter paper itself. Previous work has shown that elevated Ni concentrations in anthers are positively correlated with elevated Ni concentrations in pollen grains for *S. polygaloides* (ρ=0.61; unpublished data), thus here we use anther Ni concentrations as a surrogate for pollen Ni concentrations and as a measure of pollen quality. Nickel concentrations of pollen or nectar for each plant were used in analyses of reward quality.

6.2.5 Floral visitor observations

We arranged plants on trays for floral visitor observations, placing four Ni-treated plants and four control plants at random locations along the perimeter of a circle with a diameter of 52 cm on each tray. Two trays were placed side-by-side for observation outside at the study location. Observations were made for ten 10-minute intervals per day for 2-3 consecutive days, with the positions of the trays switched after each observation to avoid spatial bias. Two new trays of plants were observed each week for three consecutive weeks (N=48 plants; 13.33 hours of observation). Soil treatments were applied to each group of sixteen plants for the two weeks immediately prior to observation of those plants. To determine whether Ni exposure in soil affects the likelihood of flower visitation, we calculated the probability that individual plants were visited (the number of observation intervals with at least one visit divided by the total number of observation intervals). To determine whether exposure to Ni in soil alters visitation rate, we recorded the number of flowers visited per plant per observation interval, and calculated flower visitation rates as the total number of visits to each plant / number of flowers per plant / hour. Visitation rates for each plant were averaged across all observation intervals of each week, and these average values were used in analysis of visitation rates (N=24 per soil treatment). We recorded identity of flower visitors and kept records of visitation by bees and flies separately.

6.2.6 Statistical analysis

All statistical analyses were conducted using SAS version 9.3 (SAS 2012). Flower size, display size, anther mass and nectar volume were analyzed using MANOVA (PROC GLM), with soil

treatment as a fixed effect. We used mixed-model ANOVA (PROC GLM) to determine whether plants grown in high-Ni soil accumulated more Ni into floral rewards than control plants, with soil treatment, reward type (i.e., nectar or pollen) and their interaction as fixed effects, and week as a random factor. We also used mixed-model ANCOVAs (PROC GLM) to determine whether Ni-treatment altered the probability of visitation or visitation rate, with floral visitor type (bee or fly), treatment (Ni or control) and their interaction as fixed effects, and week as a random factor. Flower size and the number of open flowers per plant were included as covariates in the ANCOVAs on visitation to account for effects of morphological variation. For all mixed-models, *F*-values were calculated by dividing the mean square of each fixed effect by the mean square of the interaction between that fixed effect and the random factor (i.e., week). Post-hoc Tukey tests were used to compare Ni concentrations in each tissue type (i.e., anther or pollen) across treatments and weeks, as well as to compare floral visitation for each floral visitor type (i.e., fly or bee) across treatments and weeks. To improve normality of residuals, visitation rate was square-root transformed and Ni concentration was natural-log transformed prior to analysis.

6.3 RESULTS

6.3.1 Floral measurements

MANOVA revealed no significant effect of Ni-treatment on any component of floral morphology or reward quantity (Wilks' λ=0.89; $F_{4,43}$=1.3; P=0.28). Plants produced similar: (1) numbers of open flowers (control: 18.71 [± 2.53]; Ni-treated: 18.25 [± 2.29]), (2) sized flowers

(control: 44.28 [± 5.01] mm; Ni-treated: 44.85 [± 5.93] mm), (3) nectar volume (control: 0.95 [± 0.1] uL; Ni-treated: 0.90 [± 0.13] uL) and (4) anther mass (control: 2.2 [± 0.7] mg; Ni-treated: 2.5 [± 1.1] mg).

6.3.2 Floral reward analysis

Plants grown in Ni-supplemented soil accumulated more Ni into floral rewards than control plants, with the difference in Ni concentration between Ni-treated plants and controls greater in pollen (400%) than nectar (100%) (Fig. 11; Table 15). As a result Ni concentrations were approximately three times greater in pollen than in nectar (Fig. 11; Table 15). While mean Ni concentrations were similar for Ni-treated plants in the first two weeks of the experiment, plants in the third week had significantly (27%) higher Ni concentrations overall (i.e., in pollen and nectar).

6.3.3 Floral visitor observations

Experimental plants were visited by two groups of floral visitors- small bees in the genus *Lasioglossum* (Halictidae) and flies in the Syrphidae family. Overall, soil treatment did not affect the probability of visitation by either insect group (Fig. 12a). However, the probability of visitation to plants varied nearly two fold among weeks (0.16-0.25), and a significant pollinator type by week interaction was found (Table 16), indicating a temporal component to variation. Specifically, the overall probability of visitation by bees was 90% lower in week one relative to weeks two and three, and the overall probability of visitation by flies was 50% higher in week

one relative to weeks two and three. There was no effect of flower number or flower size on the probability of visitation (Table 16). Per flower visitation rate to Ni-treated plants was ~50% lower than control plants (Fig. 12b; Table 17), and this effect was equivalent for bees and flies (Table 17). There was no effect of week or floral display on visitation rate, though a significant pollinator type by week interaction was found, again indicating a temporal component to visitation (Table 17). Specifically, visitation rate by bees was 60% lower in week one relative to weeks two and three.

6.4 DISCUSSION

Short-term exposure to elevated soil Ni did not alter floral display or reward quantity, but it did lead to elevated Ni concentrations in nectar and pollen of the Ni hyperaccumulator *S. polygaloides*. Our results also suggest that naïve floral visitors are unable to discriminate between Ni-treated and control flowers prior to flower visitation but instead respond to differences in floral reward chemistry following arrival at plants by visiting fewer flowers containing Ni.

The effects of soil metals on plant-animal interactions are most often considered in the context of metal hyperaccumulating plants, whose above-ground tissue metal concentrations are generally greater than 1,000 ppm dry weight (van der Ent et al. 2013). In addition to this study, previous experiments have shown periodic Ni nitrate solution treatments result in Ni hyperaccumulation to leaves, but lower metal levels in pollen and nectar of *S. polygaloides* (unpublished data). Here we build on these results to show that Ni concentrations in pollen and

nectar well below values established as hyperaccumulator thresholds alter plant-flower visitor interactions. While we directly measured Ni concentrations in anthers, rather than pollen, previous work suggests that pollen Ni accumulation would still be >100 ppm in our experimental plants (unpublished data), a concentration in floral rewards already known to alter floral visitor foraging (Meindl and Ashman 2013). Our findings corroborate recent evidence that metal accumulation, defined as tissue metal concentrations >20 or >100 ppm dry weight, depending on the metal (Reeves and Baker 2000), alter plant-insect interactions (reviewed in Mogren and Trumble 2010). For example, metal concentrations below hyperaccumulator thresholds are known to alter feeding behavior of fruit flies, as copper and cadmium concentrations in artificial diets below 1,000 ppm have resulted in feeding deterrence (Bahadorani and Hilliker 2009). In addition, arsenic accumulation has been shown to deter herbivory by grasshoppers at concentrations as low as 46 ppm in leaf tissue (Rathinasabapathi et al. 2007). Furthermore, insects feeding on tissues with relatively low metal concentrations have displayed decreased survival, including diamondback moths (Coleman et al. 2005) and armyworms (Cheruiyot et al. 2013) when fed diets containing <1,000 ppm cobalt, copper, Ni, and zinc, among others. These studies suggest that metal concentrations below hyperaccumulator thresholds may alter many plant-insect interactions, while ours is among the first to indicate plant-flower visitor interactions are also affected by metal accumulation.

Our study also found a temporal component to metal accumulation and floral visitation, as Ni accumulation was highest in the third week of the experiment, and floral visitation varied throughout. However, because we did not observe significant interactions between soil treatment and week, or a three-way interaction between soil treatment, week and floral visitor type for either the probability of visitation or visitation rate, differences in visitation across weeks are not

likely related to differences in Ni accumulation over time. While it is unclear why plants in the third week accumulated higher concentrations of Ni relative to plants in weeks one and two, this pattern may be explained by increased transpiration rates and subsequent Ni uptake, as mean temperatures in week 3 (81°F) were elevated relative to those of week one (75°F) and two (72°F). Regardless of temporal variation in Ni accumulation, Ni treatment produced a similar effect on floral visitation across weeks, in which visitation rates were consistently reduced to plants treated with Ni.

The observed decrease in floral visitation to Ni-treated plants in this study suggests floral visitors may be responding to differences in floral reward chemistry, i.e., Ni concentration. However, the mechanism by which insects perceive Ni-rich floral rewards is uncertain. Deterrence effects have been observed for insects feeding on Ni-rich vegetative tissue (Boyd et al. 2002) as well as other metals (reviewed in Vesk and Reichman 2009), but it is unclear whether deterrence occurs through taste perception or other mechanisms. For example, some studies suggest that insect herbivores feeding on metal-rich leaf tissue are deterred via post-ingestional mechanisms rather than initial taste perception (Behmer et al. 2005). While honeybees possess fewer gustatory receptors relative to other insects, such as fruit flies (10 vs. 68, respectively; de Brito Sanchez 2011), they can still detect a wide variety of compounds in nectar (de Brito Sanchez 2007), and determining pollinator abilities to detect metals will provide a broader understanding of the pollination ecology of metal accumulating plants.

Metal accumulation into floral rewards has implications for the use of hyperaccumulating plants in phytoremediation. Recently, researchers have brought to light the potential ecological and environmental consequences of phytoremediation, not all of which are positive (Gerhardt et al. 2009). For example, several species of selenium (Se) hyperaccumulators have been proposed

for use in phytoremediation of Se-contaminated soils (Zhu et al. 2009). However, considering recent findings of selenium toxicity to bees (Hladun et al. 2012), the use of insect pollinated flowering plants for phytoremediation may be detrimental to foraging pollinators. Because metal accumulators may transfer toxic metals to higher trophic levels, such as pollinators, selecting the appropriate plant species for use in phytoremediation is vital. For example, selecting wind-pollinated plants, many of which show potential as phytoremediators (Chen et al. 2004), may limit risks to insect pollinators in metal-contaminated areas.

Our results also suggest that metal hyperaccumulation in natural populations alters plant-pollinator interactions. In this study, generalist floral visitors exposed to Ni-accumulating plants were naïve to Ni-rich resources, as no plants in western PA are known to accumulate Ni in high concentrations. However, recent surveys of insect communities associated with natural populations of *S. polygaloides* in CA suggest that this species hosts a distinct floral visitor community compared to closely related sympatric, non-accumulating plant species (unpublished data). Taken together this suggests that floral metal accumulation may promote specialization by pollinators tolerant to metal-rich resources. For example, one hypothesis proposed for the function of toxic alkaloids in nectar is to favor specialist pollinators (reviewed in Adler 2001), as not all generalist pollinators would be able to tolerate relatively high concentrations of secondary compounds present in floral resources. Considering recent findings of floral visitor deterrence in response to Ni in nectar (Meindl and Ashman 2013; this study), Ni accumulation in natural populations may also have important consequences for patterns of pollen transfer and ecological specialization. For example, plant secondary compounds in nectar have been shown to alter pollinator foraging and behavior (Gegear et al. 2007), and specifically impact patterns of pollen transfer (Irwin and Adler 2008). Our study suggests that metals in floral rewards may result in

similar effects, and warrants further study of the pollination ecology of metal accumulating plants.

Table 15. Results from mixed-model ANOVA on Ni accumulation into pollen and nectar (Reward Type- RT) collected from *Streptanthus polygaloides* plants grown in either Ni-supplemented or control soils (Soil Treatment- ST). Week was included as a random factor. Bold values indicate a significant difference (*P*<0.05).

Source of variation	Num. df	Den. df	MS	*F*	*P*
Week	2	84	0.64	5.05	**0.0085**
ST	1	2	52.09	1440.31	**<0.001**
RT	1	2	1.89	26.97	**0.035**
ST x RT	1	2	9.53	77.23	**0.013**
ST x Week	2	84	0.0036	0.28	0.75
RT x Week	2	84	0.07	0.55	0.58
ST x RT x Week	2	84	0.12	0.97	0.38
Error			0.13		

Table 16. Results from mixed model ANCOVA on the probability of visitation by bees and flies (Visitor Type- VT) to *Streptanthus polygaloides* plants grown in either Ni-supplemented or control soils (Soil Treatment- ST). Week was included as a random factor. Flower size and flower number were included as covariates. Bold values indicate a significant difference ($P<0.05$).

Source of variation	Num. df	Den. df	MS	*F*	*P*
Week	2	82	0.57	3.56	**0.033**
ST	1	2	0.09	8.15	0.1
VT	1	2	0.0043	0.01	0.92
ST x VT	1	2	0.0036	0.21	0.69
ST x Week	2	82	0.012	0.75	0.49
VT x Week	2	82	0.43	27.07	**<0.0001**
ST x VT x Week	2	82	0.017	1.07	0.35
Flower Size	1	82	0.0069	0.44	0.51
Flower Number	1	82	0.12	0.74	0.39
Error			0.016		

Table 17. Results from mixed model ANCOVA on visitation rate (visits/flower/hour) by bees and flies (Visitor Type- VT) to *Streptanthus polygaloides* plants grown in either Ni-supplemented or control soils (Soil Treatment- ST). Week was included as a random factor. Flower size and flower number were included as covariates. Bold values indicate a significant difference ($P<0.05$).

Source of variation	Num. df	Den. df	MS	*F*	*P*
Week	2	82	0.068	2.65	0.077
ST	1	2	0.43	138.14	**0.0072**
VT	1	2	0.17	0.24	0.67
ST x VT	1	2	0.0028	0.54	0.53
ST x Week	2	82	0.0031	0.12	0.89
VT x Week	2	82	0.7	27.32	**<0.0001**
ST x VT x Week	2	82	0.0051	0.2	0.82
Flower Size	1	82	0.014	0.56	0.46
Flower Number	1	82	0.0019	0.08	0.78
Error			0.026		

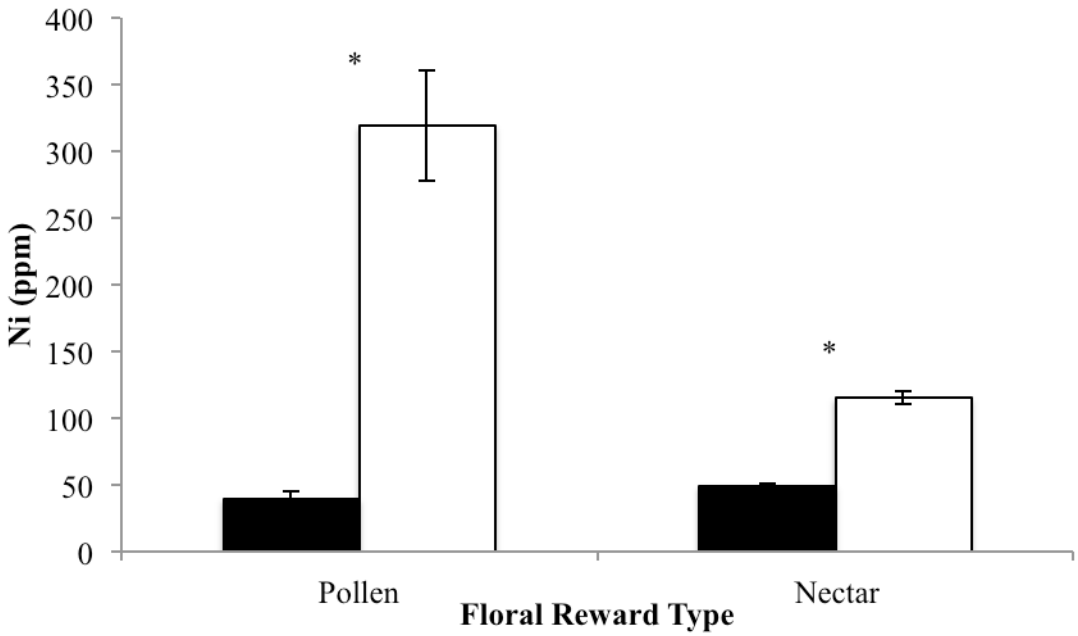

Figure 11. Nickel concentrations (ppm) in pollen and nectar samples collected from *Streptanthus polygaloides* plants (*N*=24 samples per reward type per treatment). Black symbols represent plants grown in control soil, while white symbols represent plants grown in Ni-supplemented soil. Within a floral reward type, asterisks indicate a significant difference between treatments (*P*<0.001).

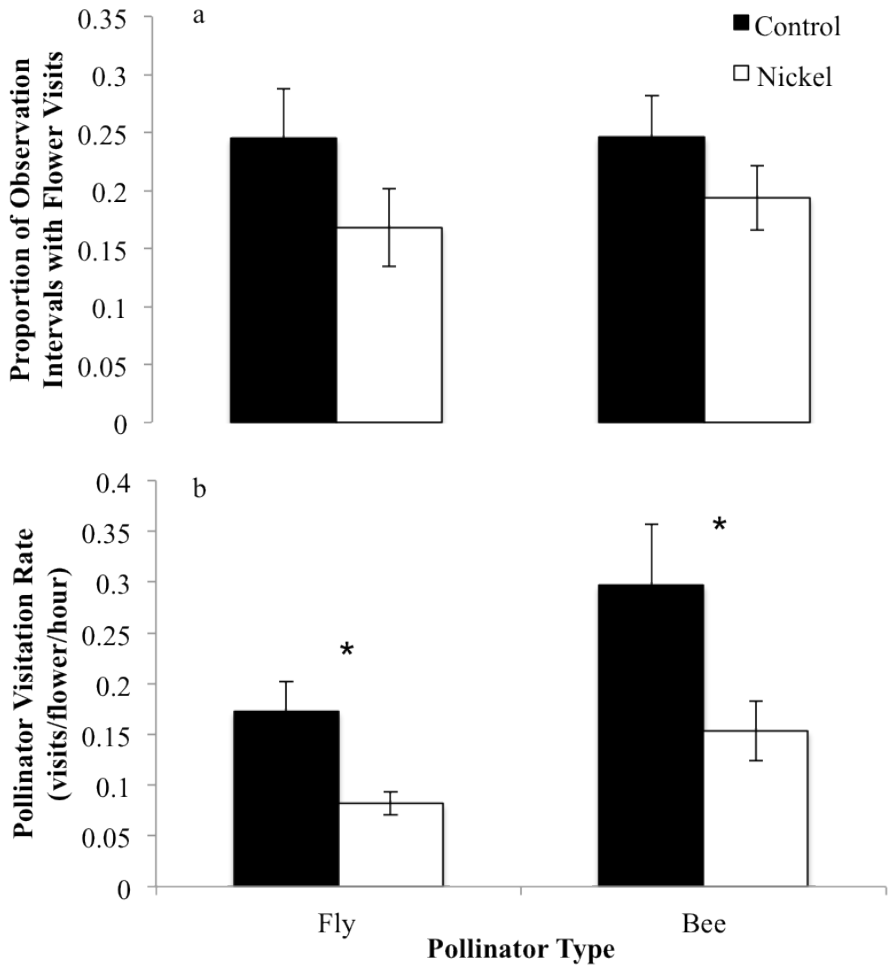

Figure 12. (a) Proportion of observation intervals in which visitation to *Streptanthus polygaloides* was observed and (b) mean visitation rates by floral visitor type (fly, bee) to *S. polygaloides* plants in experimental arrays (*N*=48 plants observed over 80 10-minute observation intervals). Black symbols represent plants grown in control soil, while white symbols represent plants grown in Ni-supplemented soil. Within a floral visitor type, bars with asterisks are significantly different between treatments (*P*<0.05).

7.0 EFFECTS OF FLORAL METAL ACCUMULATION ON FLOWER VISITOR COMMUNITIES: INTRODUCING THE 'ELEMENTAL FILTER HYPOTHESIS'

7.1 INTRODUCTION

Understanding how abiotic factors can spur reproductive isolation and speciation between closely related species is a central question in both ecology and evolution (Schluter 2009). For plants, edaphic factors are thought to play a large role in the diversification of many lineages (Rajakaruna 2004). For example, there are several mechanisms by which disparate soil environments can result in reproductive isolation between closely related taxa. Adaptation to novel soil environments can lead to species-level changes in floral morphology or phenology that can lead to reproductive isolation between sister species (Bomblies 2010). However, growth in novel soils can also lead to chemical changes in floral tissues and pollinator rewards (e.g., nectar and pollen; Meindl et al. 2013; Meindl et al. 2014a,b), which could alter pollinator visitation (Johnson et al. 2006; Meindl and Ashman 2013, 2014) and contribute to reproductive isolation between closely related plant species.

For instance, in addition to sugars, pollinator rewards often contain several minor constituents. Plants that grow on heavy metal-rich soil can accumulate metals (e.g., cadmium (Cd), nickel (Ni), and zinc (Zn), reviewed in Krämer 2010; van der Ent et al. 2013) into pollinator rewards (Meindl et al. 2014a,b), which can deter pollinators (Meindl and Ashman

2013, 2014). Likewise, floral nectars can contain microbes and secondary defensive compounds, both of which have been shown to either positively (Herrera et al. 2013; Wright et al. 2013) or negatively (Vannette et al. 2013; Adler and Irwin 2005) affect pollinator visitation. While the presence of non-nutritive compounds (e.g., secondary compounds or heavy metals) in nectar and pollen may be merely a side effect of their production in other tissues where they provide benefit, i.e., defense against herbivores or microbial pathogens (reviewed in Adler 2000), there are also possible adaptive explanations. For instance, the 'pollinator fidelity hypothesis' states that secondary compounds in nectar increase the number of quality visits from pollinators that are tolerant of 'toxic' nectar (i.e., able to consume without negative fitness effects; Boyd 2009), and reduce visits by less efficient, less tolerant generalist floral visitors (reviewed in Adler 2000). However, the idea that heavy metals may have similar effects when incorporated into floral rewards has not previously been considered. Given the deterrent effects of floral metals on generalist or naive pollinators, e.g., bumble bees and syrphid flies in some systems (Meindl and Ashman 2013, 2014), changes in floral chemistry following colonization of novel soils may act as an 'elemental filter' of floral visitors and contribute to reproductive isolation via indirect effects on plant-pollinator interactions.

Thus, we formulate a related hypothesis for the effect of plant metal hyperaccumulation on insect pollinators: the 'elemental filter hypothesis'. Here, metal-rich nectar and pollen rewards act as an 'elemental filter' that limits floral visitors to those that are unresponsive or tolerant of ingesting the metals. This hypothesis would apply to the wide range of metal hyperaccumulating plants for which leaf tissues are defended against insect herbivory via both metal toxicity (Boyd and Martens 1994) and deterrence (Kazemi-Dinan et al. 2014). In contrast, the effects of metal-rich pollen and nectar on pollinators have received far less attention (Strauss and Boyd 2011).

Yet metal-tainted rewards are likely to affect several aspects of plant-pollinator interactions (Meindl and Ashman 2013, 2014).

Specifically, heavy metals in floral rewards may act as an elemental filter on pollinators in three ways. First, metal-rich rewards could deter promiscuous or generalist pollinators because they sample flowers widely but are discriminating as they can taste or smell the metals and are repelled by them, and consequently avoid visiting metal-tainted flowers (Meindl and Ashman 2013, 2014). This would lead to reduced visitation rates by generalists (and/or overall) to a metal accumulating species. Second, taxa-specific selective deterrent effects of floral metals (Boyd and Martens 1999; Jhee et al. 2005) could translate into fewer taxa (including the generalists) visiting flowers of hyperaccumulators relative to non-accumulator species, and thus lower species richness in the flower visitor community. Third, if some insect species are tolerant, unresponsive, or even requiring of the metals in floral rewards (Boyd 2009), then there may be unique insect taxa visiting the hyperaccumulator. If community composition and/or the species richness of flower visitors differ between a hyperaccumulator and a related non-accumulator, then early stages of reproductive isolation between related plant species growing in different soil environments may be achieved (Rieseberg and Willis 2007).

As a first step towards determining whether metal hyperaccumulation acts as an elemental filter of pollinating insects, in this study we examine the pollination systems of two sympatric congeneric plant species that differ in terms of floral metal accumulation. *Streptanthus polygaloides* and *S. tortuosus* (Brassicaceae) are closely related taxa with overlapping geographic ranges (Mayer and Soltis 1994; Baldwin et al. 2012; Cacho and Strauss 2014). However, *S. polygaloides* is a Ni-hyperaccumulator that occurs exclusively on serpentine soils, typically high in Ni, while *S. tortuosus* primarily occurs on non-serpentine soils (Reeves et

al. 1981; Baldwin et al. 2012). Furthermore, the species have similar flowering times, floral morphologies (Baldwin et al. 2012) and are insect pollinated (Preston 1994; Wall and Boyd 2002). First we confirm similarities in floral display and morphology, but differences in pollinator reward Ni concentrations between species, and answer the following questions by surveying the two species in their natural populations: (1) Does the Ni hyperaccumulator *S. polygaloides* receive fewer floral visits relative to the non-accumulator *S. tortuosus*? (2) Does the composition of the pollinator pool differ between *S. polygaloides* and *S. tortuosus*? Specifically, does *S. polygaloides* host fewer floral visitor taxa, and/or is it visited by unique, unshared floral visitor taxa relative to *S. tortuosus*? Second, as an explicit test of our elemental filter hypothesis, we experimentally manipulate Ni in potted *S. polygaloides* plants to answer: (3) Is floral Ni directly responsible for altering flower visitation? (4) Is the response to high Ni floral resources more pronounced for the ambient pollinator communities residing at *S. tortuosus* than *S. polygaloides* sites?

7.2 METHODS

7.2.1 Study species

Streptanthus polygaloides and *S. tortuosus* are spring flowering herbaceous plants that are at least partially self-compatible (Meindl et al. 2014b; Wall and Boyd 2002; Preston 1994). Both species produce zygomorphic, urceolate (i.e., urn-shaped) flowers that offer both pollen and nectar to pollinators (Preston 1994; Meindl and Ashman 2014). Pollinator visitation is required

for sexual reproduction in *S. polygaloides* (Boyd et al. 2009), and is known to increase both fruit and seed production in *S. tortuosus* (Preston 1994). Floral visitors to both species are mainly bees, but also wasps, flies, beetles, and butterflies (Wall and Boyd 2002; Preston 1994). *Streptanthus tortuosus* is a perennial species that occurs throughout California and Oregon, while *S. polygaloides* is an annual species restricted to the Sierra Nevada of northern California (Baldwin et al. 2012).

7.2.2 Study sites

We studied *S. polygaloides* in three serpentine sites and *S. tortuosus* at three non-serpentine sites during May-June in 2012 and 2013. All study sites (POLY1, POLY2, POLY3, TORT1, TORT2, TORT3) were located in mixed-forest openings in the foothills of the Sierra Nevada in northern California, and were separated by five to 28 km (Fig. 13). Mean soil Ni concentrations at the serpentine sites (total Ni = 2340 ± 115 mg kg^{-1}; phytoavailable Ni = 69 ± 22 mg kg^{-1}) were elevated relative to the non-serpentine sites (total Ni = 54 ± 2 mg kg^{-1}; phytoavailable Ni = 0.15 ± 0.03 mg kg^{-1}; GAM and TLA, unpublished data).

7.2.3 Characterizing pollination systems of study species

7.2.3.1 Floral morphology and chemistry

In 2012, for each species we measured three floral traits known to be important for pollinator visitation: flower depth, flower width, and the number of flowers per inflorescence (Hegland and Totland 2005; Stang et al. 2006). We recorded the number of flowers per inflorescence on

individual plants at 3 m intervals along randomly placed transects at each site of each species (10 plants per site, 60 plants total). We then collected two flowers per plant (whose trait values were later averaged), and stored them in 70% ethanol until measuring traits at the end of the field season. Because of the urn-shaped calyx, the depth of the calyx and the width of the opening into the flower (i.e., the throat) determine the accessibility of nectar at the base of the flowers. Therefore, we measured calyx length and the width of the flower throat (mm) for flowers of each species using digital calipers.

We used mixed-model ANOVA (PROC MIXED; SAS 2012) to compare the number of flowers per inflorescence, flower depth, and flower width between species. Plant species was treated as a fixed effect, while site (nested within species) was treated as a random effect.

To verify the Ni concentrations in pollinator rewards in 2012 we collected anthers and nectar from freshly opened flowers during peak flowering. Anther and nectar samples (10 plants per site, 60 plants total) were collected from plants described above. For each plant sampled, we collected 60 anthers from unopened buds, which were air dried for 48 hours and then weighed to the nearest 0.0001 g on a AE200 Mettler® analytical balance (Mettler-Toledo, LLC, Columbus, OH, USA). We collected nectar using filter paper wicks (Whatman® Grade 1, GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) from four flowers per plant. We determined concentrations of Ni in anthers (mg kg^{-1}) and nectar (μL L^{-1}) via ICP-MS at the University of Pittsburgh, following Meindl and Ashman (2014).

We used mixed-model ANOVA (PROC MIXED; SAS 2012) to compare Ni accumulation into floral rewards between species. Plant species, reward type (i.e., anthers or nectar) and their interactions were included in the model as fixed effects, while site and

individual plant (both nested within species) were treated as random effects. We natural log transformed Ni concentration data in anthers and nectar to improve normality of residuals.

7.2.3.2 Floral visitation rate

To determine whether floral visitation rate differed between *S. polygaloides* and *S. tortuosus*, we conducted floral observations across three days per site per species in May-June 2012. We observed floral visitors during five-minute intervals at 12 sampling points between the hours of 10 a.m. and 4 p.m. on sunny days (36 observation intervals [9 hours] per site, 216 observation intervals [36 hours] total across all sites). At each sampling point, we established a 1 m^2 plot and recorded the number of flowers/plot. For each observation interval, we calculated visitation rate as the number of visits/flower/hour.

We used mixed model ANOVA (PROC MIXED; SAS 2012) to determine whether floral visitation rate was lower to *S. polygaloides* relative to *S. tortuosus*. We treated plant species as a fixed factor, and site (nested within species) as a random factor. We included flower number (per plot) and time of day of observations as covariates, to account for effects of morphological variation and temporal changes in pollinator abundance, respectively. Denominator degrees of freedom for analysis of visitation rate, as well as for all subsequent mixed models presented in this study, were determined using the Kenward-Roger approximation, which is preferred for mixed model analysis with relatively small sample sizes (Bell et al. 2014). We natural log transformed visitation rate data prior to analysis.

7.2.3.3 Ambient and flower visitor community richness and composition

To determine whether the species richness and community composition of floral visitors associated with each plant species differed and whether this was due to differences in floral visitor availability, or in visitation preferences of insects, we estimated floral visitor richness and composition via two survey methods in 2012. First, we surveyed ambient floral visitor richness and composition (hereafter, 'ambient' richness and composition) of each community using colored bowl traps (e.g., Saunders and Luck 2013). At each site, we placed nine colored plastic bowls (three each of white, blue and yellow) along a linear transect in groups of three, with each group separated by 10 m and bowls within groups separated by one m. We left bowls at each site for a total of two weeks during peak flowering, and we collected insects from bowls three times per week. Additionally, we determined flower visitor richness and composition by collecting floral visitors directly from flowers of each plant species (hereafter, 'flower visitor' richness and composition). We collected a minimum of 100 floral visitors from each species/site over the course of at least three days/site. All insects collected (from both ambient and flower visitor surveys) were later identified to species or morphospecies at the end of the field season. Vouchers of collected insects have been deposited at the Carnegie Mellon Natural History Museum for future reference.

To estimate and compare species richness of ambient and flower visitor communities, we constructed species accumulation curves using the program EstimateS (Version 9; Gotelli and Colwell 2008; Colwell 2013). We calculated the expected asymptotic richness of both ambient and flower visitor communities for each species combining across sites (Chao1; Colwell 2013), and assessed differences in estimated richness between species based on overlap of associated Chao1 95% confidence intervals (Colwell 2013). In addition, we used hierarchical,

agglomerative cluster analysis using abundance-based Bray-Curtis dissimilarity indices calculated across sites using the 'vegdist' function in 'vegan' (R Development Core Team 2012). We used the 'pvclust' function to obtain statistical support for each cluster via bootstrapping (*N* = 1000 replications; approximately unbiased probability values ≥ 95% indicate clusters of similar species composition; Suzuki and Shimodaira 2006). These results determine whether the ambient and flower visitor communities associated with each species are similar or distinct. If species-specific flower visitor communities are determined by ambient community composition, then we expect similar clustering of insect communities by plant species (i.e., ambient and flower visitor) and we also expect many shared flower visitor taxa between the two plant species. Conversely, if flower visitor communities are influenced by floral metal accumulation rather than ambient pollinator pool, we expect flower visitor communities to be dissimilar between plant species, i.e., contain few shared taxa, whereas the ambient community composition for each species will be similar.

7.2.4 Experimental test of the elemental filter hypothesis

7.2.4.1 Generation and treatment of experimental plants

Seeds of *S. polygaloides* collected from population POLY1 were germinated on a thin layer of perlite (~6 mm) in 27 in^3 pots (Deepots, Stuewe and Sons, Inc., Tangent, OR, USA) filled with potting soil (Fafard #4, Sun Gro Horticulture, Agawam, MA, USA) in a greenhouse at the University of Pittsburgh in 2013. After four weeks of growth, 80 plants were allocated to one of two Ni treatments: Ni-treated or control. Nickel was applied to Ni-treated plants by top-watering with 40 mL of Ni nitrate ($Ni(NO_3)_2$) solution containing 400 mg kg^{-1} Ni (as in Meindl et al.

2014b). Control plants were top-watered with 40 mL of ammonium nitrate (NH_4NO_3) solution to compensate for additional nitrogen (190 mg kg^{-1}) in the Ni treatment. We treated plants once per week for eight weeks and then transported them to the field sites in northern CA. Once at the field sites, we treated plants every day for two weeks prior to use in the experiment.

7.2.4.2 Floral chemistry

For each plant used in the experiment we collected 60 anthers from unopened buds and nectar from four flowers per plant and determined concentrations of Ni in anthers (mg kg^{1}) and nectar (μL L^{-1}) as above. We natural log transformed Ni concentrations in anthers and nectar to improve normality of residuals.

To verify that Ni-treated plants accumulated more Ni into floral rewards than control plants, we conducted an ANOVA (PROC GLM; SAS 2012) with soil treatment (Ni-treatment or control), reward type (anthers or nectar) and their interaction as fixed effects.

7.2.4.3 Floral visitors

To determine whether floral metals are directly responsible for altering flower visitation, and whether the response to high Ni floral resources is more pronounced for pollinator pools residing at *S. tortuosus* than *S. polygaloides* sites, in May of 2013 we observed mixed arrays of Ni-treated and control *S. polygaloides* plants. We observed experimental arrays for five days at each of two *S. polygaloides* and two *S. tortuosus* sites. We made observations at one *S. polygaloides* and one *S. tortuosus* site each day, and the order (morning vs. afternoon) was reversed on consecutive days. On each day at a given site, we observed two groups of four plants, with each group being composed of two Ni-treated and two controls arranged in a circle with a 52 cm diameter. We

observed plants for twelve five-minute intervals, and switched positions of the two groups after each interval to remove any spatial bias. We did not reuse plants following observations. We recorded visits by major functional groups (bees, beetles, and flies) separately and calculated visitation rate (v/flower/hr) by functional group for each plant, averaging across all observation intervals of each day at each site. These average values were used in analysis of visitation rates (N = 20 per soil treatment x site type; N = 80 total). Prior to floral visitor observations, we measured inflorescence height and counted the number of open flowers per plant at the start of the day for use as covariates in statistical analyses. We natural log transformed visitation rate data to improve normality of residuals.

We used a mixed model ANOVA (PROC MIXED; SAS 2012) to determine whether Ni-treatment altered floral visitation rate, with soil treatment (Ni-treated or control), floral visitor type (bee, beetle or fly) and site type (*S. polygaloides* vs. *S. tortuosus*), as well as their interactions, as fixed effects, and site (nested within site type) as a random effect. To explore significant interactions we used the SLICE option, which partitions interactions of factors so that each factor (main effect or, in the case of a significant three-way interaction, the two-way interaction term) can be tested at different levels of the other factor (Schabenberger and Pierce 2002; SAS Institute 2011). To control for variation due to floral display and temporal variation in pollinator abundance we included inflorescence height, flower number and time of observation as covariates.

Least squares means and standard errors are reported throughout.

7.3 RESULTS

7.3.1 Characterizing pollination systems of study species

7.3.1.1 Floral morphology and chemistry

The *Streptanthus* species did not differ in either floral display size (Species: $F_{1,4} = 1.1$, $P > 0.05$; *S. tortuosus* vs. *S. polygaloides*: 23.3 ± 4 vs.17.3 ± 4 flowers per inflorescence) or flower width (Species: $F_{1,4} = 0.06$, $P > 0.05$; *S. tortuosus* vs. *S. polygaloides*: 2.25 ± 0.08 vs. 2.23 ± 0.08 mm). However, *S. tortuosus* flowers were 35% deeper than *S. polygaloides* flowers (Species: $F_{1,4} = 328.85$, $P < 0.0001$; *S. tortuosus* vs. *S. polygaloides*: 7.71 ± 0.08 vs. 5.69 ± 0.08 mm).

In natural populations, *S. polygaloides* accumulated higher concentrations of Ni compared to *S. tortuosus* (Species: $F_{1,4} = 1{,}686.27$, $P < 0.0001$), though both species accumulated higher concentrations of Ni into anthers relative to nectar (Reward Type: $F_{1,58} = 887.34$, $P < 0.0001$). However, the magnitude of the difference between species varied by reward type (Species x Reward Type: $F_{1,58} = 12.96$, $P < 0.001$). Specifically, *S. polygaloides* hyperaccumulated Ni (i.e., >1,000 mg kg^{-1}) into anthers and accumulated Ni (i.e., >100 mg kg^{-1}) into nectar. Average *S. polygaloides* Ni concentration in anthers across the three study sites was 2,203 ± 140 mg kg^{-1}, while average Ni concentration in nectar was 139 ± 24 mg kg^{-1}. *Streptanthus tortuosus* had dramatically lower Ni concentrations in both anthers (28 ± 4 mg kg^{-1}) and nectar (3 ± 0.5 mg kg^{-1}).

7.3.1.2 Floral visitation rate

In natural populations, floral visitation rates were 21% lower in *S. polygaloides* than *S. tortuosus*, though this difference was not statistically significant (Species: $F_{1,4} = 5.97$, $P = 0.07$; *S. tortuosus* vs. *S. polygaloides*: 1.4 ± 0.2 vs.1.1 ± 0.2 v/flower/hr). Time of observation ($F_{1,208} = 6.63$, $P = 0.01$), but not flower number ($F_{1,208} = 0.17$, $P > 0.05$), significantly influenced visitation rates.

7.3.1.3 Ambient and flower visitor community richness

Rarefaction analysis indicated that the ambient insect communities were similar in species richness (49 for *S. polygaloides* sites compared to 46 for *S. tortuosus* sites; Fig. 14A,B; Table 18), as the 95% confidence intervals for the Choa1 estimates overlap substantially (*S. polygaloides* vs. *S. tortuosus*: 37.05-76.33 vs. 40.46-87.24; Fig. 14A,B). Similarly, rarefaction analysis of flower visitor communities indicated that the two *Streptanthus* species host similar numbers of floral visitor species (46 for *S. polygaloides* compared to 36 for *S. tortuosus*; Fig. 14C,D; Table 18), as the 95% confidence intervals for the Choa1 estimates overlap substantially (*S. polygaloides* vs. *S. tortuosus*: 33.5-94.24 vs. 30.25-40.9; Fig. 14C,D).

7.3.1.4 Ambient and flower visitor community composition

Ambient insect communities associated with *S. polygaloides* were not more similar to each other than they were to ambient insect communities associated with *S. tortuosus*, and no clusters received bootstrap support above 95% (Fig. 15A). Thirty-six potential floral visitor taxa were collected from ambient communities at *S. tortuosus* sites, while 32 potential floral visitor taxa were collected from ambient communities at *S. polygaloides* sites (Table 18). The most frequently collected taxa belonged to the insect orders Coleoptera, Diptera, and Hymenoptera

(Table 18; Fig. 15A). Fifteen taxa were collected from ambient communities at both *S. tortuosus* and *S. polygaloides* sites (Table 18). Nine of these shared taxa were bees, including honey bees (*Apis mellifera*), bumble bees (*Bombus vosnesenskii*), and a number of short-tongued bee species (*Dialictus* and *Halictus* spp.), while the remaining shared taxa were beetles, flies and ants (Table 18).

Nonetheless, the flower visitor communities associated with each plant species were distinct. *Streptanthus tortuosus* and *S. polygaloides* each hosted 31 flower visitor taxa (Table 18), ten of which were collected from both plant species (Table 18). The majority of these flower visitors were bees, as 24 bee taxa visited the flowers of *S. tortuosus* and 26 bee taxa visited *S. polygaloides* (Table 18). Bees from the family Apidae were most commonly collected on the flowers of *S. tortuosus*, including honey bees (*Apis mellifera*), several species of bumble bees (*Bombus melanopygus*, *B. vandykei*, and *B. vosnesenskii*), and carpenter bees (*Xylocopa californica* and *X. tabaniformis*; Table 18). While other members of the Apidae were observed to visit the flowers of *S. polygaloides* (e.g., *Anthophora* sp. and *Melissodes* sp.), honey bees, bumble bees, and carpenter bees were conspicuously absent (Table 18). The most commonly collected bee taxa from the flower visitor communities of *S. polygaloides* were members of the Megachilidae, including four species in the genus *Ashmeadiella* and the species *Dianthidium dubium* (Table 18), all of whom were absent from the flower visitor communities of *S. tortuosus*. Overall, only seven bee taxa were observed to visit flowers of both species (Table 18).

Cluster analysis showed that flower visitor communities associated with *S. polygaloides* are more similar to each other than they are to flower visitor communities associated with *S. tortuosus*, a topology receiving strong bootstrap support (≥ 99%; Fig. 15B). Bray-Curtis dissimilarity values ranged from 0.19-0.26 for *S. polygaloides* flower visitor community

comparisons across *S. polygaloides* sites, while values ranged from 0.19-0.54 for *S. tortuosus* flower visitor community comparisons across *S. tortuosus* sites (Fig. 15B). Comparison between the two clusters formed by *S. polygaloides* and *S. tortuosus* flower visitor communities produced a Bray-Curtis dissimilarity value close to 1, indicating limited floral visitor sharing across species (Fig. 15B).

7.3.2 Experimental test of the elemental filter hypothesis

7.3.2.1 Floral chemistry

Nickel-treated plants accumulated 177 times more Ni into floral rewards than control plants (520.15 ± 17.6 vs. 2.87 ± 17.7 mg kg^{-1}, respectively; Treatment: $F_{1,151}$ = 3,403.3, P < 0.0001). Across both treatments, Ni concentration of anthers was more than four times greater than nectar (428.57 ± 17.6 vs. 94.44 ± 17.7 mg kg^{-1}, respectively; Reward Type: $F_{1,151}$ = 154.8, P < 0.0001). Nickel concentration was 259 times greater in anthers (853.86 ± 23.6 vs. 3.28 ± 23.6 mg kg^{-1}) and 72 times greater in nectar (186.44 ± 23.6 vs. 2.45 ± 23.9 mg kg^{-1}) of Ni-treated plants than controls (Treatment x Reward Type: $F_{1,151}$ = 54.3, P < 0.0001).

7.3.2.2 Floral visitation rate

Flower visitor response to Ni-treated *S. polygaloides* was functional group- and site type-specific (Treatment x Site Type x Floral Visitor Type: P = 0.002; Table 19; Fig. 16). Specifically, visitation rate by bees was decreased by 60% in Ni-treated plants relative to controls at *S. tortuosus* sites ($F_{1,177}$ = 5.06, P < 0.05), but not at all at *S. polygaloides* sites ($F_{1,176}$ = 0.08, P > 0.05; Table 19; Fig. 16). Visitation by all other insect groups (flies and beetles) was unaffected

by Ni-treatment at either site type. Inflorescence height, flower number, and time of observation did not affect visitation rates (Table 19).

7.4 DISCUSSION

In situ S. polygaloides hyperaccumulated Ni into anthers and accumulated Ni into nectar, in stark contrast to *S. tortuosus*, the non-accumulator. In natural populations, *S. polygaloides* received lower visitation rates per flower, and attracted a different composition of floral visitors than *S. tortuosus*, from a similar ambient pool. Furthermore, experimental Ni-treatment of *S. polygaloides* reduced floral visitation by bees, but only from the *S. tortuosus* ambient pollinator pool. Together, these results indicate that not only does floral Ni accumulation lead to reduced bee visitation rates, but also that metal hyperaccumulation acts as a filter to the pollinator community leading to differences in flower visitor community composition between hyperaccumulating and non-accumulating taxa. Below, we first discuss our results in the context of the three elemental filter predictions. We then conclude by considering how floral display and morphology can influence pollinator filtering and how metal accumulation may filter other plant mutualists.

7.4.1 Predictions of the elemental filter hypothesis

First, we predicted that metal-rich floral rewards would deter generalist pollinators (Meindl and Ashman 2013, 2014) and that this would translate to lower floral visitation rates for metal

hyperaccumulating taxa. While we did observe lower floral visitation rates in natural populations to the Ni hyperaccumulating taxon, *S. polygaloides*, relative to the non-accumulator, *S. tortuosus*, this difference was not significant. However, we did observe decreased visitation rates by bees to Ni-treated *S. polygaloides* plants at *S. tortuosus* sites in our manipulative experiment (Fig. 16). As previous research has found Ni in floral rewards reduces visitation by generalist pollinators (e.g., *Lasioglossum* spp. and syrphid flies: Meindl and Ashman 2014; *Bombus impatiens*: Meindl and Ashman 2013), taken together these data suggest that floral metal accumulation reduces visitation rates by deterring some generalist pollinator taxa. Our second prediction was that taxa-specific selective deterrent effects of floral metals would translate into fewer taxa visiting flowers of hyperaccumulators relative to non-accumulators. However, we did not observe lower flower visitor richness on the hyperaccumulating species compared to the non-accumulating species. In fact, flower visitor richness was predicted to be slightly higher for the Ni-hyperaccumulating species *S. polygaloides* compared to *S. tortuosus* (Fig. 14C,D). It is important to note that while we recorded floral visitation to each plant species, we did not directly determine whether visitors were effective pollinators. Infrequent floral visitors collected from *S. polygaloides* flowers could represent promiscuous, generalist floral visitors within the community that do not regularly pollinate the flowers of this species. For example, 15 of the flower visitor taxa collected at *S. polygaloides* sites were rarely observed at flowers and were represented by no more than one individual collected per site. Thus, the possibility remains that the number of consistent, effective pollinators may differ between hyperaccumulating and non-accumulating species. However, given that each species hosted approximately the same number of flower visitor taxa in natural populations, here we conclude that floral metal accumulation does not reduce flower visitor richness for *Streptanthus* species.

Our third prediction was that *S. polygaloides* would be visited by unique, unshared floral visitor taxa relative to *S. tortuosus.* Overall, approximately 80% of observed flower visitor taxa were unshared between species. For bees, specifically, seven taxa were observed to visit both plant species, while *S. polygaloides* hosted 19 unique taxa and *S. tortuosus* hosted 17 unique taxa. Several species of generalist pollinators, including *Apis mellifera*, *Bombus* spp., and *Xylocopa* spp., were extremely abundant on *S. tortuosus* flowers, yet completely absent from those of *S. polygaloides* (Table 18). Notably, *Apis mellifera* and *Bombus* spp. were collected from the ambient community at *S. polygaloides* sites (Table 18), indicating that the insect species were present, but avoided the flowers of the hyperaccumulator. Furthermore, most of the unique bee taxa observed on *S. polygaloides* occur in genera with known floral specialists (e.g., *Dianthidium*, *Ashmeadiella*: Wilson et al. 2010; *Perdita*, *Melissodes*, *Anthidium*, *Hoplitis*, *Anthophora*: Griswald et al. 1997), whereas all of the bee taxa collected solely from *S. tortuosus* occur in genera generally known to form broad floral associations (*Apis, Bombus, Ceratina, Dialictus, Halictus, Osmia, Xylocopa*). In addition to differences in flower visitor community composition, the observed reduction in bee visitation rates to Ni-treated *S. polygaloides* plants only at *S. tortuosus* sites suggest that some bee species found in *S. polygaloides* sites are unresponsive or tolerant to Ni-rich floral resources, and hence show no preference between Ni-treated and control plants. Thus, we provide novel evidence that metal hyperaccumulating plants host unique species of pollinating insects, relative to closely related plants occurring in sympatry. Because floral metal accumulation may result in pollinator filtering, closely related plant species occurring in sympatry that differ in floral metal accumulation may become reproductively isolated. Previous studies have found correlations between edaphic shifts and pollination system shifts for plant sister species with overlapping geographic ranges (van der Niet et al. 2006)- our

study highlights one possible mechanism by which these shifts may occur, i.e., the elemental filter.

Surveys of insect taxa associated with metal hyperaccumulating plants are rare, but a small number of studies suggest that insect herbivores may become specialized and monophagous on metal hyperaccumulating plants. Wall and Boyd (2002) and Boyd et al. (2006) collected insect herbivores from Ni hyperaccumulating plants and analyzed them for tissue Ni concentrations. These authors found that some insect herbivores contained extraordinarily high Ni concentrations, suggesting these insects possess tolerance mechanisms that allow them to feed on high-Ni plant tissues (reviewed in Boyd 2009). Furthermore, bees collected from the flowers of metal hyperaccumulating plants contain 3-8 times more Ni in their bodies compared to bees collected from non-hyperaccumulating plant species, suggesting trophic transfer of metals from plants to pollinators (Wall and Boyd 2002; Boyd et al. 2006). Nickel accumulation in pollinators suggests that they, in addition to herbivores, may become tolerant or even specialized to metal-rich floral resources. Further studies that examine fitness costs to pollinators when fed metal-rich resources, however, would be required to determine if any of these taxa are tolerant to heavy metals (Boyd 2009).

While differences in the composition of flower visitor communities were observed between species, it is also possible that insect species may become locally tolerant to metal-rich floral rewards at the population level, rather than strict specialists at the species level. Though we did not collect any *Apis* or *Bombus* spp. from *S. polygaloides* flowers in this study, Wall and Boyd (2002) collected both *Apis mellifera* and *Bombus vandykei* from the flowers of *S. polygaloides* from populations in southern California. Furthermore, Preston (1994) collected some species of bees from *S. tortuosus* that, in this study, were only observed on *S. polygaloides*

(i.e., *Anthophora urbana* and *Dianthidium dubium*). It is therefore possible that insect species, much like ecotypic differentiation observed in plants growing in metal-rich soils (Turner et al. 2010), may become locally adapted, or tolerant, to metal-rich resources. Indeed, herbivorous moths occurring in metal-contaminated areas have shown evidence for local adaptation to heavy metals, as individuals from polluted sites grew larger when fed metal-tainted resources compared to individuals from unpolluted sites (van Ooik and Ratala 2010). Whether pollinator species may also become locally tolerant to heavy metal-rich floral rewards is as yet undetermined. Additionally, metal-rich floral rewards may be deterrent to generalist pollinators like *Apis* and *Bombus* spp. only when there are more rewarding coflowering plant species present in the community. For instance, Gegear et al. (2007) found that nectar alkaloids generally deterred visitation by bumble bees, but only when alternative floral resources of better quality (i.e., higher nectar sugar concentration and/or or lower alkaloid concentration) were provided. Therefore, in addition to population- or species-level tolerance to heavy metal-rich resources, coflowering community context may also influence the deterrent effects of heavy metals in floral rewards on generalist pollinators.

7.4.2 Considering floral display and morphology

We need to acknowledge, however, that floral traits, such as overall floral display, flower depth, and flower width, can be important in terms of filtering floral visitors (Hegland and Totland 2005) by constraining taxa from utilizing floral resources. For example, Herrera (1996) found that plant species with shorter flower tubes were visited by more floral visitor species than plants with longer flower tubes. Likewise, Stang et al. (2006) found that flower visitor richness

decreased with both increasing nectar holder depth and decreasing nectar holder width. In this study, *S. polygaloides* and *S. tortuosus* had similar floral displays (flowers per inflorescence) and flower width, but differed in flower depth. The deeper flowers of *S. tortuosus* could, in part, explain differences in *in situ* flower visitor community composition observed in this study. For example, while the difference was not significant, we did observe higher flower visitor richness to the species with shallower flowers (*S. polygaloides*), which would be predicted if this trait were filtering flower visitor communities across species (Herrera 1996; Stang et al. 2006). However, taxa of both longue-tongued (*Anthidium mormonum* and *Osmia* spp. [Megachilidae]) and short-tongued (*Dialictus* sp. [Halictidae]) bees were observed to visit the flowers of both species, suggesting that their floral morphologies were not limiting visitation to different subsets of the ambient pollinator pools. While flower visitor community composition can be affected by floral morphology, our data suggest that floral metal accumulation, which markedly differed between species and was shown to reduce bee visitation rates to experimental *S. polygaloides* plants, likely plays a larger role.

7.4.3 Extending the elemental filter to other plant mutualisms

In addition to plant-herbivore interactions, metal hyperaccumulation influences plant-mutualist interactions, including plant-mycorrhizae (reviewed in Alford et al. 2010) and plant-pollinator (Meindl and Ashman 2013; Meindl and Ashman 2014; this study) interactions. Furthermore, the tree species *Sebertia acuminate* produces fleshy fruits with pulp that contains 6,900 mg kg^{-1} Ni (Boyd et al. 2006), suggesting that seed dispersal agents may also be affected by floral metals. However, these mutualistic interactions remain understudied, thus it is unclear what the overall

fitness costs and/or benefits of metal hyperaccumulation are, and whether the elemental filter may apply to other plant mutualists. For example, one hypothesis explaining the presence of secondary metabolites in ripe fruits is that these toxic chemicals promote seed dispersal by tolerant mutualists and limit seed predation by intolerant antagonists ('directed toxicity hypothesis'; reviewed in Cipollini and Levey 1997). Similar adaptive hypotheses should be assessed for metal hyperaccumulators that produce fleshy fruits, such as *Sebertia acuminate*. Determining any possible adaptive value (e.g., elemental defense or mutualist filtering) of metal hyperaccumulation requires an understanding of both the benefits and detriments of this trait, and thus further study of plant mutualists as well as antagonists.

Table 18. Insect order, family, species and functional group for insects collected from natural populations of *Streptanthus polygaloides* and *S. tortuosus* in northern CA from both flower visitor and ambient communities in May-June 2012. 'Both' means that the insect taxon was collected at both *S. polygaloides* ('POLY') and *S. tortuosus* ('TORT') populations, while 'None' means that it was collected at neither.

Order	Family	Species	Functional Group	Flower Visitor	Ambient
Coleoptera	Melyridae	Coleoptera sp. 1	Beetle	Both	Both
Coleoptera	Melyridae	Coleoptera sp. 2	Beetle	POLY	POLY
Diptera	Callophoridae	Diptera sp. 4	Fly	None	Both
Diptera	Sarcophagidae	Diptera sp. 3	Fly	None	POLY
Diptera	Syrphidae	Diptera sp. 1	Fly	TORT	Both
Diptera	Syrphidae	Diptera sp. 2	Fly	Both	POLY
Diptera	Tachidinae	Diptera sp. 5	Fly	None	TORT
Diptera	Tachidinae	Diptera sp. 6	Fly	None	POLY
Hemiptera	Miridae	*Prepops* sp.	Other	Both	POLY
Hymenoptera	Andrenidae	*Andrena knuthiana*	Bee	None	TORT
Hymenoptera	Andrenidae	*Perdita* sp.	Bee	POLY	None
Hymenoptera	Andrenidae	*Perdita blatchleyi*	Bee	None	TORT
Hymenoptera	Apidae	*Anthophora urbana*	Bee	POLY	None
Hymenoptera	Apidae	*Apis mellifera*	Bee	TORT	Both
Hymenoptera	Apidae	*Bombus melanopygus*	Bee	TORT	None
Hymenoptera	Apidae	*Bombus vandykei*	Bee	TORT	None
Hymenoptera	Apidae	*Bombus vosnesenskii*	Bee	TORT	Both
Hymenoptera	Apidae	*Ceratina arizonensis*	Bee	Both	POLY
Hymenoptera	Apidae	*Ceratina nanula*	Bee	Both	None
Hymenoptera	Apidae	*Ceratina sequoiae*	Bee	TORT	None
Hymenoptera	Apidae	*Ceratina tejonensis*	Bee	TORT	None
Hymenoptera	Apidae	*Diadasia bituberculata*	Bee	None	POLY
Hymenoptera	Apidae	*Melissodes* sp.	Bee	POLY	None
Hymenoptera	Apidae	*Xylocopa californica*	Bee	TORT	None
Hymenoptera	Apidae	*Xylocopa tabaniformis*	Bee	TORT	TORT
Hymenoptera	Apidae	*Eucera* sp. 1	Bee	None	POLY

Hymenoptera	Apidae	*Eucera* sp. 2	Bee	None	TORT
Hymenoptera	Chrysididae	Chrysididae sp.	Bee	None	TORT
Hymenoptera	Crabronidae	*Miscophus* sp.	Bee	None	TORT
Hymenoptera	Crabronidae	*Solierella* sp.	Bee	None	POLY
Hymenoptera	Formicidae	Formicidae sp. 1	Other	TORT	Both
Hymenoptera	Formicidae	Formicidae sp. 2	Other	None	Both
Hymenoptera	Formicidae	Formicidae sp. 3	Other	None	Both
Hymenoptera	Halictidae	*Dialictus* sp. 1	Bee	Both	None
Hymenoptera	Halictidae	*Dialictus* sp. 2	Bee	TORT	None
Hymenoptera	Halictidae	*Dialictus* sp. 3	Bee	POLY	None
Hymenoptera	Halictidae	*Dialictus* sp. 4	Bee	POLY	Both
Hymenoptera	Halictidae	*Dialictus* sp. 5	Bee	POLY	None
Hymenoptera	Halictidae	*Dialictus* sp. 6	Bee	POLY	None
Hymenoptera	Halictidae	*Dialictus* sp. 7	Bee	POLY	None
Hymenoptera	Halictidae	*Dialictus* sp. 8	Bee	POLY	Both
Hymenoptera	Halictidae	*Dialictus* sp. 9	Bee	None	POLY
Hymenoptera	Halictidae	*Dialictus* sp. 10	Bee	None	Both
Hymenoptera	Halictidae	*Dialictus* sp. 11	Bee	None	Both
Hymenoptera	Halictidae	*Dialictus* sp. 12	Bee	None	POLY
Hymenoptera	Halictidae	*Halictus tripartitus*	Bee	TORT	Both
Hymenoptera	Halictidae	*Halictus farinosus*	Bee	None	Both
Hymenoptera	Halictidae	*Lasioglossum trizonatum*	Bee	POLY	None
Hymenoptera	Halictidae	*Lasioglossum* sp. 1	Bee	None	POLY
Hymenoptera	Halictidae	*Lasioglossum* sp. 2	Bee	None	POLY
Hymenoptera	Halictidae	*Micralictoides ruficaudus*	Bee	None	POLY
Hymenoptera	Megachilidae	*Anthidium mormonum*	Bee	Both	TORT
Hymenoptera	Megachilidae	*Anthidium utahense*	Bee	POLY	None
Hymenoptera	Megachilidae	*Anthidium illustre*	Bee	None	POLY
Hymenoptera	Megachilidae	*Anthidiellum notatum*	Bee	POLY	none
Hymenoptera	Megachilidae	*Ashmeadiella californica* ssp. *sierra*	Bee	POLY	None
Hymenoptera	Megachilidae	*Ashmeadiella foveata*	Bee	POLY	POLY
Hymenoptera	Megachilidae	*Ashmeadiella timberlakei*	Bee	POLY	None
Hymenoptera	Megachilidae	*Ashmeadiella* sp.	Bee	POLY	None
Hymenoptera	Megachilidae	*Chelostoma minutum*	Bee	POLY	None
Hymenoptera	Megachilidae	*Dianthidium dubium*	Bee	POLY	None
Hymenoptera	Megachilidae	*Hoplitis producta*	Bee	POLY	None
Hymenoptera	Megachilidae	*Osmia* sp. 1	Bee	TORT	None
Hymenoptera	Megachilidae	*Osmia* sp. 2	Bee	Both	None
Hymenoptera	Megachilidae	*Osmia* sp. 3	Bee	TORT	TORT
Hymenoptera	Megachilidae	*Osmia* sp. 4	Bee	Both	TORT
Hymenoptera	Megachilidae	*Osmia* sp. 5	Bee	TORT	TORT

Hymenoptera	Megachilidae	*Osmia* sp. 6	Bee	TORT	None
Hymenoptera	Megachilidae	*Osmia* sp. 7	Bee	TORT	None
Hymenoptera	Megachilidae	*Osmia* sp. 8	Bee	TORT	Both
Hymenoptera	Megachilidae	*Osmia* sp. 9	Bee	TORT	TORT
Hymenoptera	Megachilidae	*Osmia* sp. 10	Bee	None	TORT
Hymenoptera	Megachilidae	*Osmia* sp. 11	Bee	None	TORT
Hymenoptera	Megachilidae	*Osmia* sp. 12	Bee	None	TORT
Hymenoptera	Megachilidae	*Protosmia rubifloris*	Bee	Both	None
Hymenoptera	Vespidae	*Pseudomasaris vespoides*	Bee	None	POLY
Hymenoptera	Vespidae	*Odynerus* sp.	Bee	None	POLY
Lepidoptera	Hesperiidae	Lepidoptera sp. 1	Butterfly	TORT	TORT
Lepidoptera	Hesperiidae	Lepidoptera sp. 2	Butterfly	TORT	TORT
Lepidoptera	Hesperiidae	Lepidoptera sp. 3	Butterfly	None	TORT
Lepidoptera	Hesperiidae	Lepidoptera sp. 4	Butterfly	None	TORT
Lepidoptera	Lycaenidae	Lepidoptera sp. 5	Butterfly	None	TORT
Lepidoptera	Lycaenidae	Lepidoptera sp. 6	Butterfly	None	TORT

Table 19. Results from generalized linear mixed model on visitation rate (visits/flower/hour) by bees, beetles and flies (Pollinator Type) to arrays of *Streptanthus polygaloides* plants grown in either Ni-treated or control soils (Treatment) and observed in either *S. polygaloides* ('POLY') or *S. tortuosus* ('TORT') sites (Site Type). Inflorescence height, flower number and the time of observation were included as covariates.

(A) Source of variation	df (Num., Den.)	*F*	*P*
Treatment	1,116	1.6	0.21
Site Type	1,115	3.55	0.062
Pollinator Type	2,304	5.78	**0.0035**
Treatment*Site Type	1,116	0.03	0.87
Treatment*Pollinator Type	2,304	0.41	0.66
Treatment*Site Type*Pollinator Type	4,304	4.24	**0.0023**
Inflorescence Height	1,71	2.22	0.14
Flower Number	1,71	0.23	0.63
Time of Observation	1,71	3.67	0.06

(B) Slices for Treatment by Site Type*Pollinator Type				
Site Type	Pollinator Type			
TORT	Bee	1,177	5.06	**0.026**
TORT	Beetle	1,375	0	0.99
TORT	Fly	1,375	0.07	0.79
POLY	Bee	1,176	0.07	0.8
POLY	Beetle	1,375	0	0.98
POLY	Fly	1,375	1.11	0.29

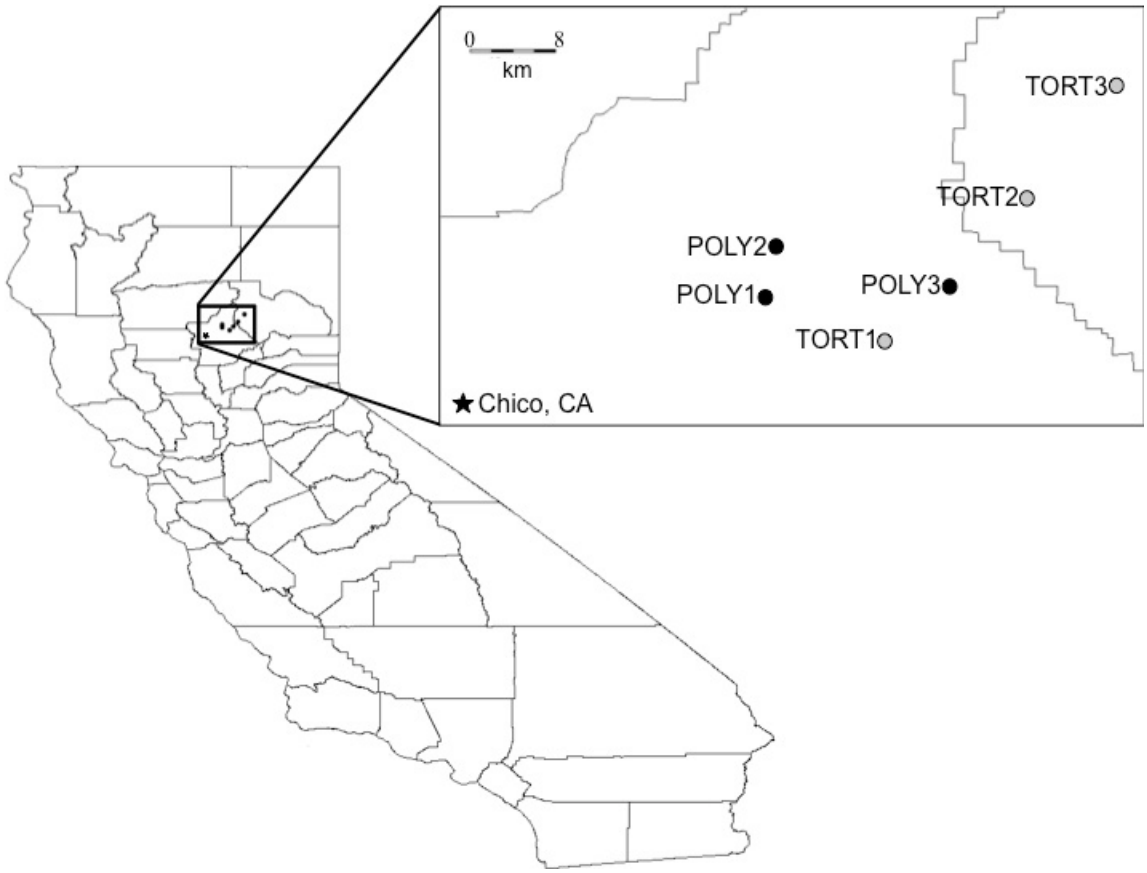

Figure 13. Map of six study sites located in northern California. Serpentine sites containing *Streptanthus polygaloides* (POLY1, POLY2, POLY3) are marked with black circles, while non-serpentine sites containing *S. tortuosus* (TORT1, TORT2, TORT3) are marked with grey circles. The northern California city of Chico (marked with a black star) is included as a reference point.

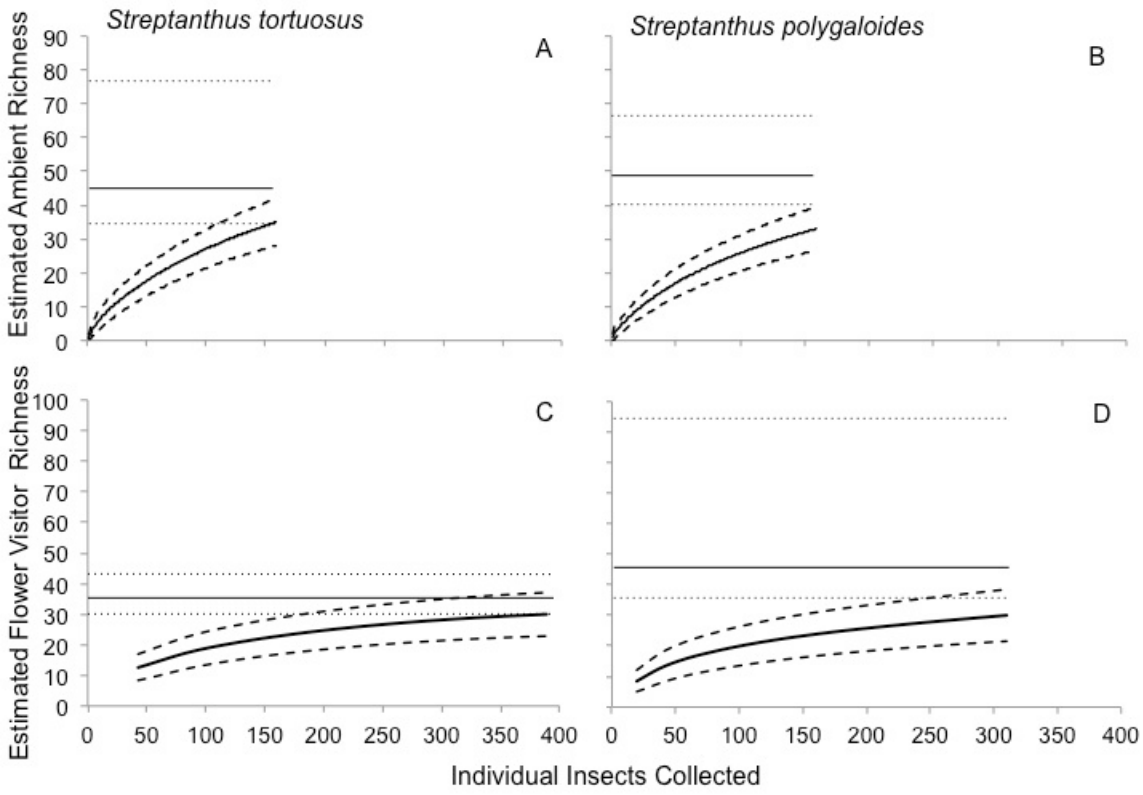

Figure 14. Rarefaction curves for estimated ambient community richness (A,B) and flower visitor richness (C,D) to *Streptanthus tortuosus* (A,C) and *S. polygaloides* (B,D). Horizontal lines represent Choa1 richness estimates (solid lines) with associated 95% confidence intervals (dashed lines).

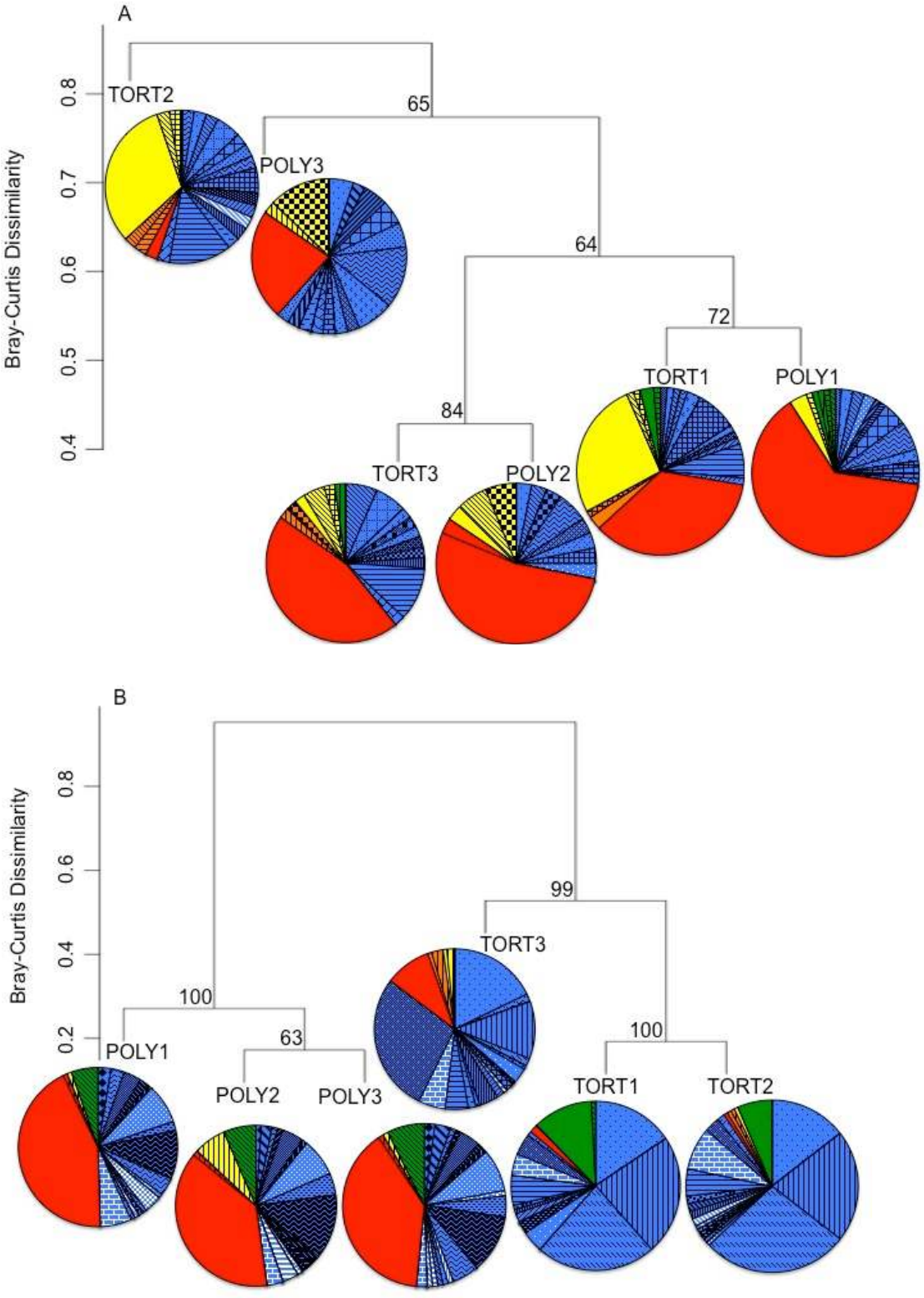
A
Bray-Curtis Dissimilarity
0.8
0.7
0.6
0.5
0.4
TORT2
POLY3
TORT3
POLY2
TORT1
POLY1
65
64
72
84
B
Bray-Curtis Dissimilarity
0.8
0.6
0.4
0.2
POLY1
POLY2
POLY3
TORT3
TORT1
TORT2
99
100
63
100

Figure 15. Results of cluster analysis comparing insect floral visitor similarity between *Streptanthus tortuosus* (TORT1-3) and *S. polygaloides* (POLY1-3) populations based on (A) ambient community composition and (B) flower visitor community composition. Values at dendrogram nodes indicate results from bootstrapping (values ≥ 95% are statistically supported clusters). Pie charts indicate the relative proportion of floral visitor types for each site collection, and taxa are represented by different colors: beetles (red), butterflies (orange), flies (yellow), 'other' visitors (e.g., ants; green), and bees (blue).

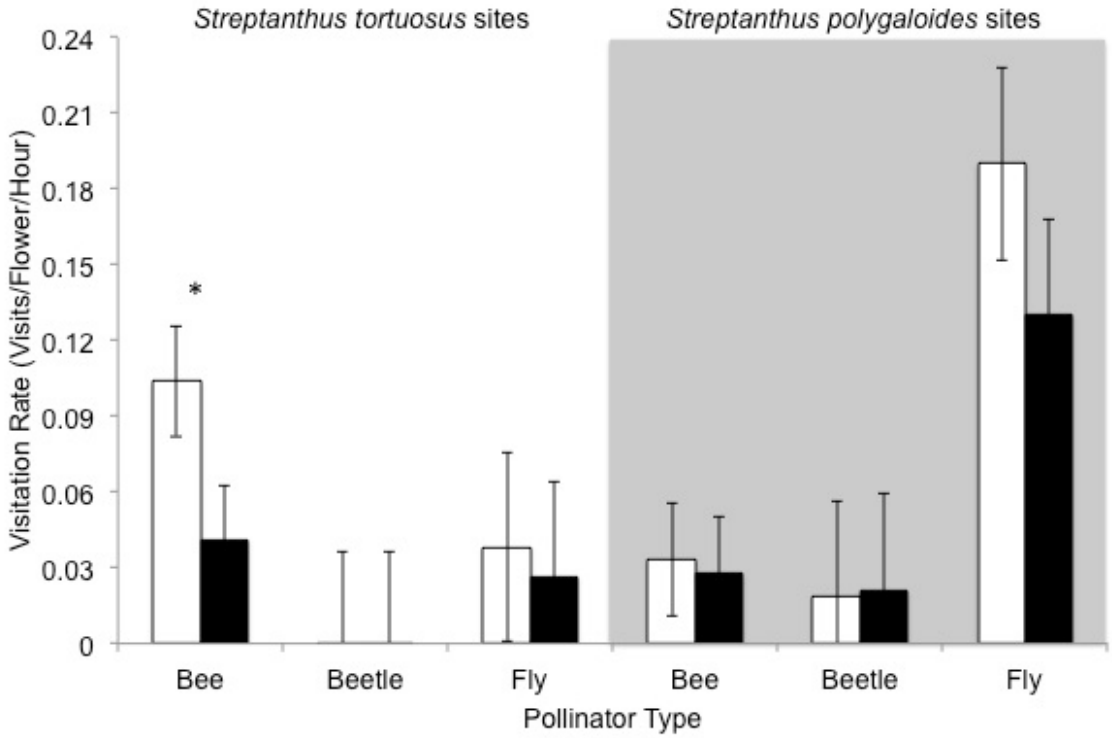

Figure 16. Mean (±SE) floral visitation rates (visits/flower/hour) by bees, beetles, and flies to control (white bars) or Ni-treated (black bars) *Streptanthus polygaloides* plants in arrays presented at *S. tortuosus* (left bars, unshaded) and *S. polygaloides* sites (right bars, shaded). Asterisks indicate differences ($P < 0.05$) between treatments.

8.0 DIFFERENTIAL EFFECTS OF SOIL CHEMISTRY ON POLLEN GERMINATION, FRUIT AND SEED PRODUCTION FOR TWO PLANT SPECIES THAT DIFFER IN SERPENTINE AFFINITY AND FLORAL METAL ACCUMULATION

8.1 INTRODUCTION

The abiotic environment is known to affect the abundance, distribution and diversification of species (Schluter 2009), and edaphic factors are an especially strong selective force for plants (Rajakaruna 2004; Anacker and Strauss 2014). Serpentine soils, which are a nutritionally stressful growing environment for most plants due to deficiencies of essential nutrients (e.g., calcium [Ca]) and high concentrations of heavy metals (e.g., nickel [Ni]; Brooks 1987; Brady et al. 2005; Kazakou et al. 2008), have long been considered model habitats to study plant reproductive isolation and speciation (Kay et al. 2011). Serpentine soils can indirectly contribute to pre- (or post-) zygotic reproductive barriers that ultimately isolate serpentine populations from non-serpentine progenitors (Kay et al. 2011). For example, adaptation to serpentine soil can lead to habitat isolation, temporal isolation (i.e., non-overlapping flowering times), and pollinator isolation (Macnair and Gardner 1998; Gardner and Macnair 2000; Hughes et al. 2001; Wright et al. 2006; Sambatti and Rice 2007; Wright and Stanton 2007). Though less studied, soil chemistry may also lead to selection at the level of gametes favoring assortative mating. In a

series of experiments with *Mimulus guttatus* (Phrymaceae), Searcy and Mulcahy (1985) and Searcy and Macnair (1990) showed that copper (Cu) in the pistils of plants could act as a selective filter since seed production was reduced when pollen donors were not tolerant to soils with elevated Cu levels. Floral heavy metal accumulation may therefore produce a prezygotic isolating mechanism by decreasing fitness when maternal and paternal plants grow in soil environments with different metal concentrations (e.g., serpentine and non-serpentine), and thus act as a reproductive barrier that promotes reproduction between plants growing in similar soil environments. However, heavy metals are not the only elements found in serpentine soils that may alter plant mating patterns. Calcium, an alkaline earth metal, is required for pollen germination and tube growth (Brewbaker and Kwack 1963). Specifically, Ca influences the direction of pollen tube growth, as pollen tubes grow chemotropically along an increasing Ca gradient from the top to the bottom of the pistil (Mascarenhas and Machlis 1962, 1964; Rosen 1968; Chichiriccò 2002). Variation in soil Ca can influence the likelihood of hybridization between species, as pollen that develops in low-Ca environments has higher fertilization success on stigmas that develop in high-Ca environments for some plant species (e.g., *Phlox cuspidata* and *P. drummondii*; Ruane and Donohue 2007). Therefore, understanding the effects of soil chemistry on floral chemistry is vital not only for identifying potential reproductive costs associated with plant growth on chemically unique soils, such as serpentine, but also for explaining patterns of species distributions, reproductive isolation and plant endemism.

Geographic regions containing serpentine soil often support many endemic species, indicating it is a potent force in speciation (Brooks 1987; Safford et al. 2005; Anacker 2011). However, plant species span a gradient of affinity to serpentine soils, with some commonly found either on or off (i.e., non-endemic) whereas others are entirely restricted to serpentine soil

(i.e., endemic; Safford et al. 2005). High Ni and low Ca concentrations, in particular, are thought to be key in generating adaptation to serpentine soil (Lazarus et al. 2011; Kazakou et al. 2008), and serpentine affinity is known to affect both Ni and Ca accumulation into plant tissues (Nagy and Proctor 1997; Burrell et al. 2012; DeHart et al. 2014; Meindl et al. 2014a). This differential acquisition of Ni and Ca between endemic and non-endemic species may lead to differences in plant fitness, as Ni is generally considered toxic to plants (e.g., Ni reduces pollen germination [Breygina et al. 2012] and seed production [Malan and Farrant 1998] in non-adapted species) and Ca is required for a variety of metabolic functions, including pollen germination (Marschner 2012). This may be particularly true for Ni, as differences in Ni accumulation between endemic and non-endemic species are most pronounced for reproductive organs (i.e., anthers and pistils), where serpentine endemics limit uptake of metals more than non-endemics (Meindl et al. 2014a). Endemics can be more efficient at extracting Ca from serpentine soils than non-endemics (DeHart et al. 2014), suggesting that non-endemics may require higher soil Ca concentrations to achieve sufficient internal concentrations. Because of the toxic effects of Ni, and the beneficial effects of Ca, differential acquisition of these elements when growing in serpentine soil may result in differential fitness between taxa due to effects on pollen germination, and subsequent fruit and seed production.

A relatively small number of serpentine taxa are known to accumulate extremely high concentrations of heavy metals (i.e., hyperaccumulation), most of which are endemic to serpentine soils (Pollard et al. 2014). Metal hyperaccumulation refers to the uptake and sequestration of soil metals by plants into above ground tissues in concentrations that are orders of magnitude higher than typical plants (reviewed in van der Ent et al. 2013). For example, hyperaccumulators of the heavy metal Ni exhibit shoot Ni concentrations >1,000 mg kg^{-1}, while

most plants contain <5 mg kg^{-1} (van der Ent et al. 2013). Several hypotheses regarding the adaptive value of metal hyperaccumulation have been suggested, including its role in elemental allelopathy, drought resistance, and defense against herbivores and pathogens (reviewed in Boyd and Martens 1992). However, direct effects on plant reproduction have rarely been characterized, thus it is difficult to assess the adaptive value of metal hyperaccumulation. A preliminary study of the Ni hyperaccumulator *Alyssum inflatum* (Brassicaceae) found that plants are more likely to flower, and produce larger floral displays, when grown in Ni-supplemented soils (Ghasemi et al. 2014). These data suggest that metal hyperaccumulators do not incur fitness costs due to growth in metal-rich soils, and may actually directly benefit from metal hyperaccumulation. However, additional studies are required that assess the direct fitness costs (or benefits) of metal hyperaccumulation in order to determine its adaptive value and relation to plant endemism on metal-rich soils.

In this study, we employ a fully factorial experiment to test for the effects of Ni and Ca on pollen germination, fruit production, and seed production for two closely related species that vary in their affinity to serpentine soil (*Streptanthus polygaloides* [serpentine endemic and a Ni hyperaccumulator]; *S. tortuosus* [non-endemic, non-accumulator]. Specifically, we asked the following question: Do soil Ni and Ca concentrations affect plant reproduction (i.e., pollen germination, fruit production, and seed production), and does this differ between species? We predict that plant reproduction will be maximized when soil treatments most closely reflect the chemical composition of the native soil. Specifically, we predict that (1) soil Ni will negatively impact plant reproduction only for *S. tortuosus*, (2) soil Ca will positively impact plant reproduction only for *S. tortuosus*, and (3) plant reproduction will be highest for *S. polygaloides*

when grown in high Ni, low Ca soil, and that plant reproduction will be highest for *S. tortuosus* when grown in low Ni, high Ca soil.

8.2 METHODS

8.2.1 Study species

Streptanthus polygaloides and *S. tortuosus* are closely related taxa (Mayer and Solstis 1994; Cacho and Strauss 2014) in the mustard family (Brassicaceae). *Streptanthus tortuosus* is common and can be found throughout California and Oregon, while *S. polygaloides* is restricted to the Sierra Nevada of northern California, where the ranges of the two species overlap (Baldwin et al. 2012). *Streptanthus polygaloides* is a Ni hyperaccumulating, annual endemic to serpentine soil (Baldwin et al. 2012; Reeves et al. 1981). *Streptanthus tortuosus* is a non-Ni hyperaccumulating perennial that can occur either on or off of serpentine soil, though it is more frequently found on non-serpentine soil (Baldwin et al. 2012). Both species are spring-flowering herbs that are at least partially self-compatible (Meindl et al. 2014b; Wall and Boyd 2002; Preston 1994).

8.2.2 Experimental design

We collected seeds from a single natural population per species in northern California (*S. polygaloides*: 39°49' N 121°34’ W; *S. tortuosus* [a non-serpentine population]: 39°51' N 121°24’ W) in the summer of 2012. In the fall of 2013, we treated seeds for two weeks with 4°C cold and

dark conditions. Following germination, we transplanted seedlings to 27 in^3 pots (Deepots, Stuewe and Sons, Inc., Tangent, OR, USA) filled with standard potting soil (Fafard #4, Sun Gro Horticulture, Agawam, MA, USA) and supplied with six Nutricote® NPK 13-13-13 time-release fertilizer pellets (Arysta LifeScience Corporation, New York, NY, USA). One month after transplanting, we subjected *S. tortuosus* plants to a 4°C cold treatment for one month at 8D:16N. Subsequently, we grew both *S. tortuosus* and *S. polygaloides* under controlled conditions of 12D:12N and between 21.1-26.7°C until flowering in the greenhouse at the University of Pittsburgh.

One month after transplanting (*S. polygaloides*), or one week after cold treatment (*S. tortuosus*), we divided plants into four treatment groups (N = 20 plants / species / treatment; total N = 160 plants): (1) high Ni and high Ca, (2) high Ni and low Ca, (3) low Ni and high Ca, or (4) low Ni and low Ca. We introduced soil treatments by top watering plants with 40 mL of treatment solution once per week for the duration of the experiment (eight weeks). High Ni solutions contained 500 mg kg^{-1} Ni, while low Ni solutions contained 5 mg kg^{-1} Ni. High Ca solutions contained 2,200 mg kg^{-1} Ca, while low Ca solutions contained 220 mg kg^{-1} Ca. Both Ni and Ca solutions were prepared using metal nitrates ($Ni[NO_3]_2$-$6H_2O$ or $Ca[NO_3]_2$-$4H_20$). We added ammonium nitrate (NH_4NO_3) to treatment solutions (2)-(4) to control for additional nitrogen applied to plants in the high Ni high Ca treatment. Our soil treatment solutions reflect realistic levels of bioavailable Ni and Ca because bioavailable fractions of Ni in serpentine soils generally range from 50 to 500 mg kg^{-1}, while those in non-serpentine soils generally range between 0.05 to 5 mg kg^{-1} (L'Huillier and Edighoffer 1996; Chardot et al. 2005; Broadley et al. 2012). Likewise, bioavailable fractions of Ca in non-serpentine soils generally range from 2,000-4,000 mg kg^{-1}, while those in serpentine soils generally range from 100-600 mg kg^{-1} (Whittaker

1954; DeHart et al. 2014). Of the 160 experimental plants, we randomly assigned half to be pollen donors, and the other half to be pollen recipients.

To determine the effect of soil treatment on plant chemistry, each week we collected pollen and pistil samples from 40 flowers pooled across all donor plants for each treatment and species separately. Previous work has determined anther Ni concentrations are positively correlated with pollen Ni concentrations for *S. polygaloides* (ρ=0.61; unpublished data), thus here we use anther Ni concentrations as a surrogate for pollen Ni concentrations. We determined Ni and Ca using Inductively Coupled Plasma Mass Spectrometry (ICP-MS, NEXION 300X, PerkinElmer, Waltham, MA, USA) at the University of Pittsburgh following Meindl and Ashman (2014), and concentrations are reported as mg kg^{-1}.

To determine the effects of donor and recipient soil treatments on pollen germination and fruit and seed production we performed hand-pollinations. For each species separately, we bulk-collected pollen from donor plants of each treatment once per week. We collected whole anthers, placed them in open microcentrifuge tubes, and allowed them to dehisce overnight. The following day, we performed pollinations on each of the recipient plants. Once per week, we pollinated four-eight flowers per recipient plant (N = 4-16 flowers pollinated per plant, N = 596 total flowers pollinated). Each flower received pollen from one of the four donor treatments in random order. For half of these flowers, we collected styles 24 hours after performing pollinations and fixed the styles in 70% ethanol. We then softened and stained styles with analine blue (Dafni 1992; Arceo-Gómez and Ashman 2011) and observed them with the aid of an epifluorescence microscope (Axioskop, Carl Zeiss Microscopy, LLC, Thornwood, NY, USA). For each style, we recorded the total numbers of pollen, germinated pollen, and pollen tubes that reached the ovary. We then calculated the percentage of pollen applied that germinated (number

of germinated pollen grains / total number of pollen grains) and the percentage of germinated pollen to reach the ovary (number of pollen tubes that reached ovary / number of germinated pollen grains). We recorded whether the remaining pollinated flowers matured fruits, and for those that did we determined the number of fertile seeds. For plants that had multiple flowers pollinated per donor treatment, we calculated average values of all responses.

We conducted statistical analyses in SAS (version 9.3; SAS Institute Inc., Cary, NC, USA). To evaluate the effect of soil donor and recipient Ni and Ca treatments on the percentage of pollen applied that germinated, the percentage of germinated pollen to reach the ovary, and seeds produced per fruit, we used mixed-model ANCOVA (PROC MIXED). To evaluate the effect of soil donor and recipient treatments on the likelihood of pollinated flowers to mature fruits, we used log-linear analysis (PROC GLIMMIX) and specified a binary distribution (SAS Institute 2011). We included the total number of pollen grains applied to stigmas as a covariate in pollen germination models. We also used mixed model ANOVA (PROC MIXED) to compare Ni and Ca concentrations in pistils and pollen across species and soil treatments, with species, soil Ni treatment, soil Ca treatment, and organ type and their interactions as fixed effects, and sampling week as a random effect. Denominator degrees of freedom for *F*-tests were determined using the Kenward-Roger approximation, which is preferred for small sample sizes and unbalanced data (Bell et al. 2012). We included individual plant ID (nested within species) as a random effect in all models.

We included terms in final statistical models that addressed specific a priori hypotheses. First, we hypothesized that soil Ni and Ca treatments will affect both paternal and maternal reproductive function, as Ni is known interfere with pollen germination and seed production, and Ca concentration in pistils is known to positively influence pollen germination (thus we included

the main effects of recipient and donor treatments: Recipient Ni, Recipient Ca, Donor Ni, Donor Ca). Second, since we hypothesized that plant species will differ in their response to soil Ni and Ca, as one species (*S. polygaloides*) is endemic to Ni-rich, Ca-poor soils (i.e., serpentine soil), while the other species (*S. tortuosus*) is not (thus we included species by recipient and donor treatment interactions: Species, Species*Recipient Ni, Species*Recipient Ca, Species*Donor Ni, Species*Donor Ca). Specifically, we expect *S. tortuosus* to respond negatively to Ni treatment in all aspects of reproduction, as it generally grows in soils with low Ni concentrations, and *S. polygaloides* to respond neutrally or positively, as it grows in Ni-rich serpentine soil and hyperaccumulates this element. Similarly, we expect *S. tortuosus* to respond positively to Ca treatment, whereas we expect no response from *S. polygaloides*, as this species typically grows in soils with little available Ca. Furthermore, we hypothesize that soil Ni and soil Ca treatments may interact, and that reproductive success for each species will be maximized when soil treatment levels most closely reflect the chemical composition of their native soils (thus we included the interactions between the two donor and recipient treatments, as well as the three way interactions between these effects and species: Recipient Ni*Recipient Ca, Donor Ni*Donor Ca, Species*Recipient Ni*Recipient Ca, Species*Donor Ni*Donor Ca). For example, we predict that *S. polygaloides* will have highest fitness (i.e., high rates of pollen germination, fruit and seed production) when plants are grown in soils with high Ni and low Ca (mimicking serpentine soil), and the opposite will be true for *S. tortuosus*, i.e., this species will have highest fitness when grown in soils with low Ni and high Ca (mimicking non-serpentine soil). We did not include 4- or 5-way interactions in the final models, but, as determined by AIC values, our reduced models based on a priori hypotheses produced a better fit for the data compared to full models (percent pollen germination model: full model AIC = -32.6, reduced model AIC = -68.2; percent of

germinated pollen to reach ovary model: full model AIC = 3, reduced model AIC = -13.3; seed set model: full model AIC = 36.4, reduced model AIC = 21.5). Furthermore, we explored various stepwise variable selection methods (i.e., forward, backward, stepwise, lasso, and lar methods) to determine which effects should be retained in final models, and all effects chosen by these stepwise variable selection methods represented those already retained due to the above stated hypotheses.

Least squares means and standard errors are reported throughout.

8.3 RESULTS

8.3.1 Pistil and pollen chemistry

Nickel treatment significantly increased Ni concentrations across pollen and pistils in both species, but the magnitude of the treatment effect varied by both species and tissue (Ni Treatment x Species x Tissue Type: $F_{1,36} = 29.1$, $P < 0.0001$). Specifically, Ni concentrations in pistils were nearly 17 times higher for plants in the high Ni treatment relative to the low Ni treatment for *S. polygaloides* (1,381.9 ± 59.8 vs. 82.2 ± 59.8, respectively), while Ni concentrations in pistils were approximately seven times higher for plants in the high Ni treatment relative to the low Ni treatment for *S. tortuosus* (188.8 ± 84.6 vs. 27.9 ± 84.6, respectively). Furthermore, Ni concentrations in pollen were nearly nine times higher for plants in the high Ni treatment relative to the low Ni treatment for *S. polygaloides* (155.5 ± 53.5 vs. 18.0 ± 53.5, respectively), while Ni concentrations in pollen were approximately 13 times higher

for plants in the high Ni treatment relative to the low Ni treatment for *S. tortuosus* (106.5 ± 84.6 vs. 8.3 ± 84.6, respectively). Despite Ca concentrations being slightly elevated overall for plants in the high Ca treatment relative to the low Ca treatment (6,711.6 ± 439.5 vs. 6.319.3 ± 439.5, respectively), these differences were not statistically significant, and none of the interactions involving treatment and species were significant either (All $P > 0.05$).

8.3.2 Pollen germination

While neither pollen recipient nor donor Ca treatments affected the pollen germination for either species, both recipient and donor Ni treatment significantly affected pollen germination, though the effects varied by species (Table 20A; Fig. 17A). Recipient Ni treatment did not strongly influence the percentage of pollen to germinate for *S. tortuosus* (high Ni: 0.67 ± 0.04, low Ni: 0.7 ± 0.04), but pollen germination was slightly elevated for plants in the high Ni treatment relative to the low Ni treatment for *S. polygaloides* (0.72 ± 0.04 vs. 0.59 ± 0.04, respectively; Species x Recipient Ni Treatment: $F_{1,59.3} = 4.19$, $P < 0.05$; Table 20A; Fig. 17A). Conversely, while donor Ni treatment did not strongly influence the percentage of pollen to germinate for *S. polygaloides* (high Ni: 0.67 ± 0.03, low Ni: 0.65 ± 0.03), pollen germination was reduced for *S. tortuosus* plants pollinated with pollen from plants in the high Ni-treatment pollen (high Ni: 0.65 ± 0.03, low Ni: 0.72 ± 0.03; Species x Donor Ni Treatment: $F_{1,194} = 3.33$, $P < 0.05$; Table 20A). The total number of pollen grains applied and plant ID also significantly influenced pollen germination (Table 20A).

Neither pollen recipient nor pollen donor Ca treatments affected the percentage of germinated pollen to reach the ovary for either species (Table 20B). However, recipient Ni

treatment significantly affected pollen germination, though the effect varied by species (Table 20B; Fig. 17B). Specifically, recipient Ni treatment reduced the percentage of germinated pollen to reach the ovary for *S. tortuosus* (high Ni: 0.44 ± 0.04, low Ni: 0.67 ± 0.04), but the percentage of germinated pollen to reach the ovary was elevated for plants in the high Ni treatment relative to the low Ni treatment for *S. polygaloides* (0.76 ± 0.04 vs. 0.62 ± 0.05, respectively; Species x Recipient Ni Treatment: $F_{1,56.9} = 17.66$, $P < 0.01$; Table 20B; Fig. 17B). The total number of pollen grains applied and plant ID also significantly influenced pollen germination (Table 20B).

8.3.3 Fruit production

Neither pollen recipient nor donor Ca treatments affected the likelihood of pollinated flowers to mature fruits (Table 1C). However, the likelihood of pollinated flowers to mature a fruit was strongly influenced by recipient Ni treatment, though the effect varied by species (Table 20C; Fig. 18). For *S. polygaloides*, 74% of flowers of recipient plants in the high Ni treatment produced fruit, while 45% of flowers produced fruit for recipient plants in the low Ni treatment. For *S. tortuosus*, 26% of flowers of recipient plants in the high Ni treatment produced fruit, while 81% of flowers produced fruit for recipient plants in the low Ni treatment (Species x Recipient Ni Treatment: $F_{1,58.8} = 24.69$, $P < 0.01$; Table 20C; Fig. 18).

8.3.4 Seed production

Calcium treatments did not affect seed production for either species (Table 20D). Recipient Ni treatment significantly affected seed production, though the effect varied by species (Table 20D;

Fig. 19). While recipient Ni treatment reduced seed production for *S. tortuosus* (high Ni: 3.5 ± 1.9, low Ni: 11.0 ± 1.4), seed production was elevated for plants in the high Ni treatment relative to recipient plants in the low Ni treatment for *S. polygaloides* (high Ni: 10.5 ± 1.3, low Ni: 7.1 ± 2.3; Species x Recipient Ni Treatment: $F_{1,47.8} = 9.4$, $P < 0.01$; Table 20D; Fig. 19). Plant ID also significantly influenced pollen germination (Table 20D).

8.4 DISCUSSION

Serpentine soils are an abiotically stressful growing environment for plants, in large part due to low available Ca and elevated Ni concentrations (Brady et al. 2005; Kazakou et al. 2008). While both elements in isolation are known to alter pollen germination and fruit and seed production (Ruane and Donohue 2007; Breygina et al. 2012), ours is the first study to simultaneously test for effects of both elements on pollen germination, fruit production, and seed production for species that are known to vary in serpentine affinity and floral metal accumulation. While Ca may generally be important for pollen grain germination and tube growth, our study suggests that the elevated Ni concentrations in serpentine soil are more likely to affect plant reproduction for non-endemic species and thus limit their ability to reproduce on these harsh soils. In addition, our study suggests that soil metals may increase the reproductive potential of metal hyperaccumulating plants, adding new insights to the potential adaptive value of this trait.

While Ca is known to be an important nutrient for plant reproduction (Brewbaker and Kwack 1962), in this study we did not observe an effect of soil Ca treatment on pollen germination, fruit production, or seed production. Because adequate Ca concentration in plant

tissues ranges between 1,000 and 50,000 mg kg^{-1} (White 2003), and tissue Ca concentrations were above 6,000 mg kg^{-1} across all treatments in this study, Ca may not have been limiting for plants in any of the treatments. Furthermore, Ca and Ni may compete for uptake by plant roots, as studies have documented both negative and positive correlations between the accumulations of these two elements by plants (Robinson et al. 1999; Chaney et al. 2008). Though Ni treatment did not significantly influence tissue Ca concentrations (Ni Treatment: $F_{1,31.6} = 2$, $P = 0.16$), Ca concentrations were elevated in plants that received the high Ni treatment relative to plants that received the low Ni treatment (6,902 ± 439.5 vs. 6,128.87 ± 439.5 mg kg^{-1}, respectively). Similarly, though Ca treatment did not significantly influence tissue Ni concentrations (Ca Treatment: $F_{1,36} = 2.32$, $P = 0.14$), Ni concentrations were elevated in plants that received the high Ca treatment relative to plants that received the low Ca treatment (285 ± 36 vs. 207 ± 36 mg kg^{-1}, respectively). Therefore, any potential beneficial effects of increased tissue Ca concentrations could have been negated by increased Ni concentrations. As previous studies have shown soil Ca to possibly be important in preventing reproductive isolation between species growing in adjacent soil environments that differ in Ca concentrations (Ruane and Donahue 2007), further studies are needed that document the effects of Ca on plant reproductive success for serpentine tolerant species.

Our results suggest that floral metal accumulation may contribute to reproductive isolation between serpentine and non-serpentine plant populations. While several speciation models have been proposed based on geographic proximity of diverging populations, the allopatric model of speciation is the most widely accepted (Felsenstein 1981; Coyne and Orr 2004). However, serpentine soils provide a model to test whether parapatric speciation may occur, as they are often distributed as islands embedded within a non-serpentine matrix (Harrison

and Inouye 2002; Kay et al. 2011) and many plant species are able to grow both on and off of serpentine (i.e., serpentine tolerant; Harrison and Inouye 2002; Safford et al. 2005). For serpentine and non-serpentine populations to become reproductively isolated, barriers must exist to prevent gene flow- results from this study suggest that floral metal accumulation may provide a mechanism through which gene flow is reduced between serpentine and non-serpentine populations. Specifically, we found that *S. tortuosus* plants grown in high-Ni soils displayed decreased pollen germination relative to plants grown in low-Ni soils. This suggests that pollen arriving from a non-serpentine plant is unlikely to be successful in siring progeny, as the Ni concentrations in the pistils of maternal plants may limit pollen grain germination and/or pollen tube growth towards ovules. While soil heavy metals have been previously implicated in fostering reproductive isolation between populations on vs. off metal-rich soil indirectly via changes in floral phenology (Antonovics 2006), our study provides evidence for a more direct mechanism through which soil metals may impart reproductive isolation between populations. Similar to what has been observed for the metal Cu in *Mimulus guttatus* (Searcy and Mulcahy 1985; Searcy and Macnair 1990), floral Ni accumulation may provide a selective barrier to gene exchange between serpentine and non-serpentine populations, and thus provide a prezygotic isolating mechanism between populations that vary in floral metal concentrations and/or metal tolerance.

Our results provide further evidence that the elevated heavy metal concentrations of serpentine soils present a significant barrier to plant colonization for non-adapted plant species (Brady et al. 2005; Kazakou et al. 2008). While some plants require small concentrations of Ni as an active component of the enzyme urease (Welch 1981), Ni in excess is known to negatively impact plant reproduction, such as decreasing pollen germination (Tuna et al. 2002; Breygina et

al. 2012) and seed production (Malan and Farrant 1998) for plants not adapted to soils with elevated Ni. Here, we found that both paternal and maternal components of plant reproduction were inhibited for *S. tortuosus*, a non-endemic serpentine species, when grown in Ni-rich soils. Specifically, pollen from *S. tortuosus* plants grown in Ni-treated soils displayed decreased germination rates relative to pollen from plants grown in low-Ni soils. Furthermore, *S. tortuosus* plants grown in Ni-treated soils were less likely to produce fruits, and the fruits that were produced contained fewer seeds, relative to plants grown in low-Ni soils. Therefore, non-serpentine plants that are not adapted or tolerant to the high heavy metal concentrations of serpentine are unlikely to successfully reproduce on serpentine soils, as Ni accumulation into reproductive organs limits overall plant fitness. Limiting floral metal accumulation is likely a key adaptation to plant growth on serpentine soils for endemic plant species that are not known to hyperaccumulate heavy metals (DeHart et al. 2014; Meindl et al. 2014a).

Results from this study suggest that metal hyperaccumulators may directly benefit from metal hyperaccumulation in terms of increased fitness. There are several hypotheses regarding the adaptive value of metal hyperaccumulation, including defense against herbivores and pathogens, and elemental allelopathy against other plant species (reviewed in Boyd and Martens 1992). However, recent research has suggested that hyperaccumulating species may achieve higher fitness when grown in metal-rich soils, suggesting a more direct benefit of increasing metal concentrations in aboveground tissues. For example, *Alyssum inflatum* (Brassicaceae), a Ni hyperaccumulator native to serpentine soils in Anatolia, was more likely to flower, and produced more inflorescences and more flowers, when grown in Ni-supplemented soils compared to Ni-free control soils (Ghasemi et al. 2014). In another Ni hyperaccumulating species, *Alyssum murale* (Brassicaceae), germination rates for seeds produced by maternal plants grown in high-

Ni soils were twice as high compared to seeds derived from parents grown on low-Ni soils (M. McKenna, pers. comm). Similarly, reproductive benefits have been documented for the selenium (Se) hyperaccumulator *Stanleya pinnata* (Brassicaceae), as pollen germination was observed to be higher when germinated on artificial media that contained Se, relative to media that did not (Prins et al. 2011). In this study, we found that the Ni hyperaccumulator *S. polygaloides* exhibited higher rates of pollen germination, was more likely to produce fruit, and produced more seeds per fruit when maternal plants were grown in high-Ni soils relative to low-Ni soils. The mechanism that produced these patterns is unknown, but may relate to the beneficial effects of Ni on plant N metabolism (Polacco et al. 2013). Regardless of the mechanism, however, our study provides additional evidence of the beneficial effects of metal hyperaccumulation on plant reproduction for metal hyperaccumulating species (Ghasemi et al. 2014). Patterns such as those demonstrated in this study may help to explain why the vast majority of metal hyperaccumulating plants are entirely restricted, i.e., endemic, to soils with elevated metal concentrations (Pollard et al. 2014). Specifically, our study suggests that metal hyperaccumulators achieve higher fitness when grown in metal-rich soils, suggesting that they are specifically adapted to these environments (i.e., the specialist model of edaphic endemism; Meyer 1980; Palacio et al. 2007). A growing number of studies are finding that hyperaccumulating plants concentrate hyperaccumulated elements into reproductive tissues (Quinn et al. 2011; Meindl et al. 2014b), which may directly increase their fitness and help explain their patterns of distribution, often exclusively on metal-rich soils.

Soil chemistry may greatly influence plant reproduction in chemically unusual soils, such as serpentine. Soil chemistry may affect plant reproduction indirectly by altering pollinator visitation (Meindl and Ashman 2014), flowering phenology (Gardner and Macnair 2000;

Antonovics 2006), or flower morphology (Gardner and Macnair 2000; Meindl et al. 2013), but there may also be direct effects that result from altered floral tissue chemistry (Searcy and Mulcahy 1985; Searcy and Macnair 1990; this study). Further study of effects of soil chemistry on floral chemistry, and subsequent effects on gamete function and compatibility, will help elucidate the role of unique soils in fostering local adaptation, speciation and endemism in plants.

Table 20. Results from mixed models on (A) the percentage of pollen that germinated, (B) the percentage of germinated pollen that reached the ovary, (C) the likelihood of pollinated flowers to produce fruit, and (D) the number of seeds produced per fruit for *S. polygaloides* and *S. tortuosus* (Species) plants that were grown in one of four recipient soil treatments (RecipientNi, RecipientCa) and whose flowers were pollinated with pollen that came from plants grown in one of four donor soil treatments (DonorNi, DonorCa). The total number of pollen grains applied (Total Grains) was included as a covariate in models (A) and (B).

	(A) Percentage of Pollen that Germinated		**(B) Percentage of Germinated Pollen that Reached Ovary**		**(C) Likelihood to Produce Fruit**		**(D) Seeds per Fruit**	
Fixed Effects	***df***	***F***	***df***	***F***	***df***	***F***	***df***	***F***
Total Grains	1, 258	**14.81****	1, 257	**24.49****	NA	NA	NA	NA
Species	1, 68.2	0.66	1, 65	**9.56****	1, 58.7	0.33	1, 47.8	0.72
RecipientNi	1, 61.2	1.84	1, 58.6	0.94	1, 58.8	1.33	1, 47.8	1.34
RecipientCa	1, 58.9	0.33	1, 56.6	0.71	1, 58.7	3.16	1, 47.8	0.02
DonorNi	1, 195	1.58	1, 192	0.21	1, 438	0.39	1, 137	0.04
DonorCa	1, 195	3.71	1, 193	0.06	1, 438	0.3	1, 136	0.06
Species*RecipientNi	1, 59.3	**4.19***	1, 56.9	**17.66****	1, 58.8	**24.69****	1, 47.8	**9.4****
Species*RecipientCa	1, 58.8	0.11	1, 56.5	0.54	1, 58.7	1.64	1, 47.8	0.46
Species*DonorNi	1, 194	**5.33***	1, 192	3.05	1, 438	0.44	1, 137	0.14
Species*DonorCa	1, 196	1.44	1, 193	0.36	1, 438	1.95	1, 136	0.02
RecipientNi*RecipientCa	1, 59.1	0.05	1, 56.8	0.08	1, 58.7	0.47	1, 47.8	0.99
DonorNi*DonorCa	1, 194	0.94	1, 191	0.76	1, 438	0.01	1, 136	3.35
Species*RecipientNi*RecipientCa	1, 58.8	0.01	1, 56.5	0.01	1, 58.7	2.09	1, 47.8	0.06
Species*DonorNi*DonorCa	1, 195	0.48	1, 192	1.67	1, 438	0.29	1, 136	1.36
Random Effect		***Z***		***Z***		***Z***		***Z***
Plant ID (Species)		**3.79****		**3.94****		NA		**3.85****

***P<0.05; **P<0.01**

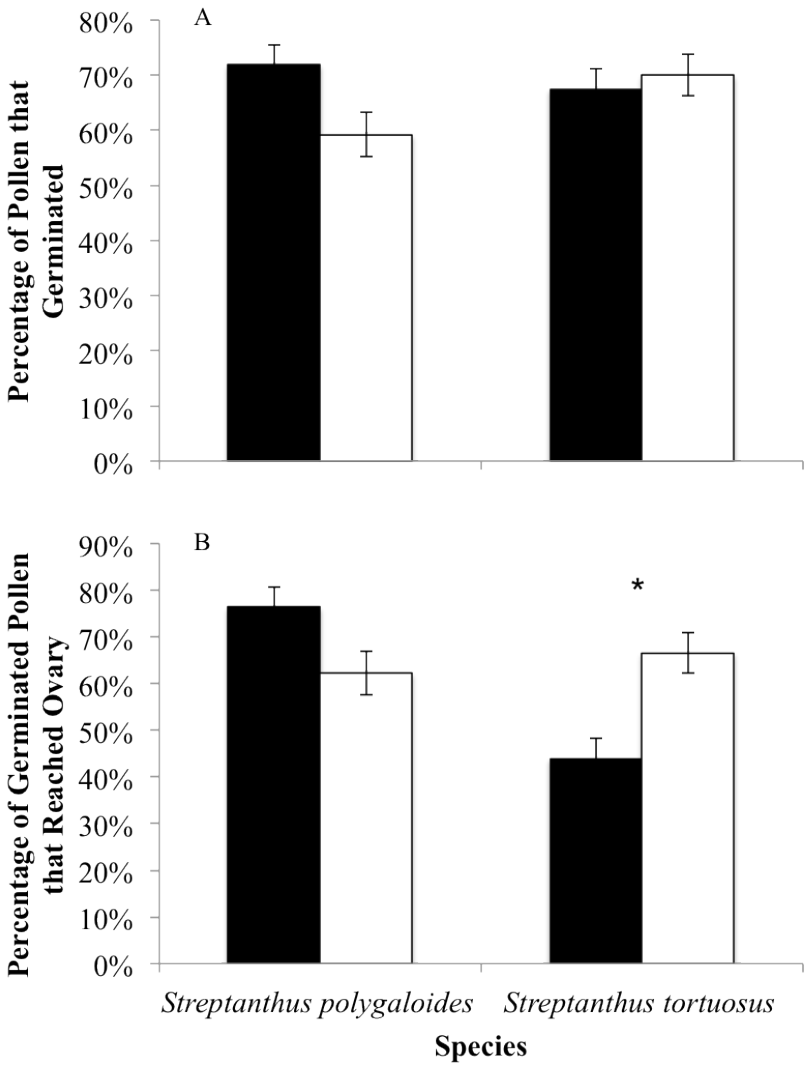

Figure 17. The percentage of pollen that germinated (A) and the percentage of germinated pollen that reached the ovary (B) for *Streptanthus polygaloides* and *S. tortuosus* plants that were either grown in Ni-treated soils (black bars) or soils that were not Ni-treated (white bars). Bars represent lsmeans and associated standard errors. Asterisks indicate significant (i.e., adjusted *P*-value < 0.05) pairwise Tukey tests.

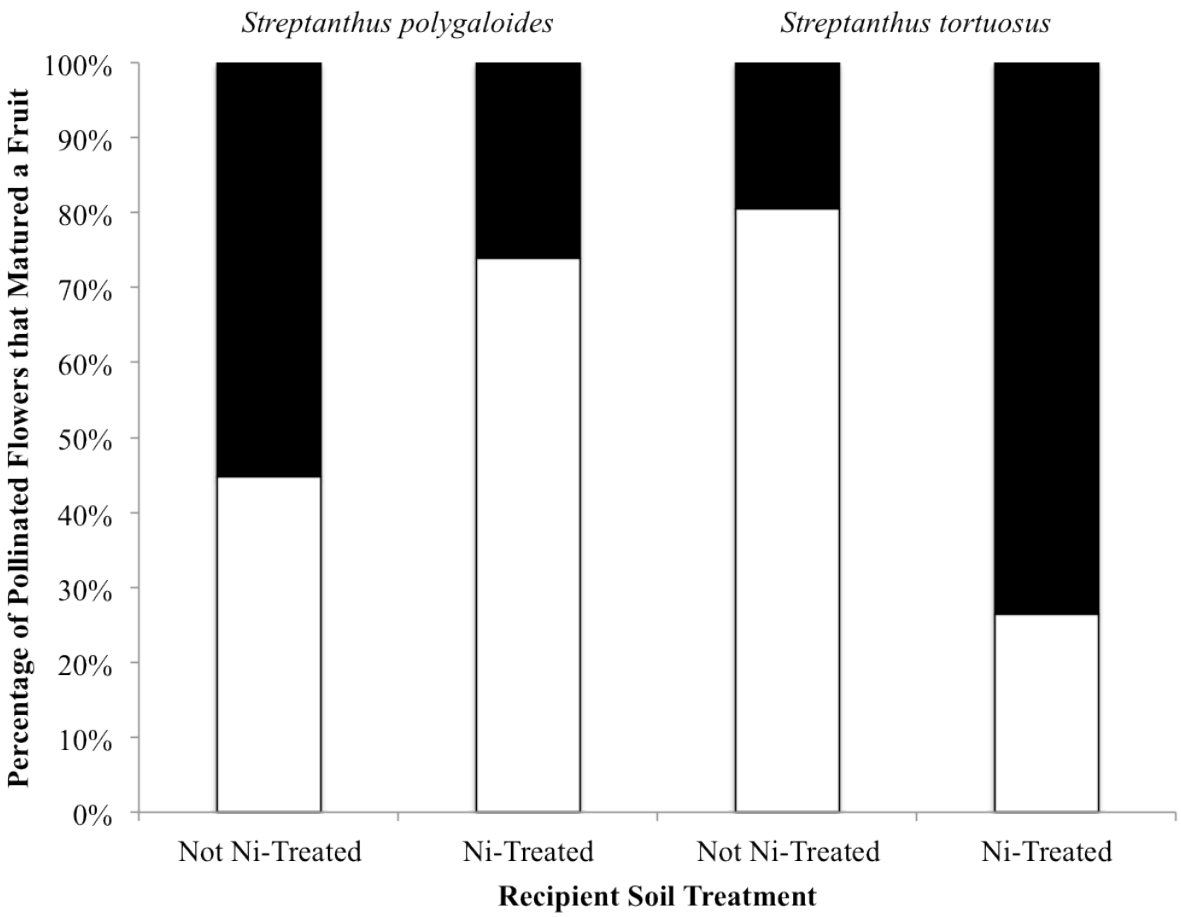

Figure 18. The percentages of pollinated flowers that matured fruits for *Streptanthus polygaloides* and *S. tortuosus* plants that were either grown in Ni-treated soils or soils that were not Ni-treated. White portions of bars represent flowers that matured a fruit, whereas black sections of bars represent flowers that did not mature a fruit.

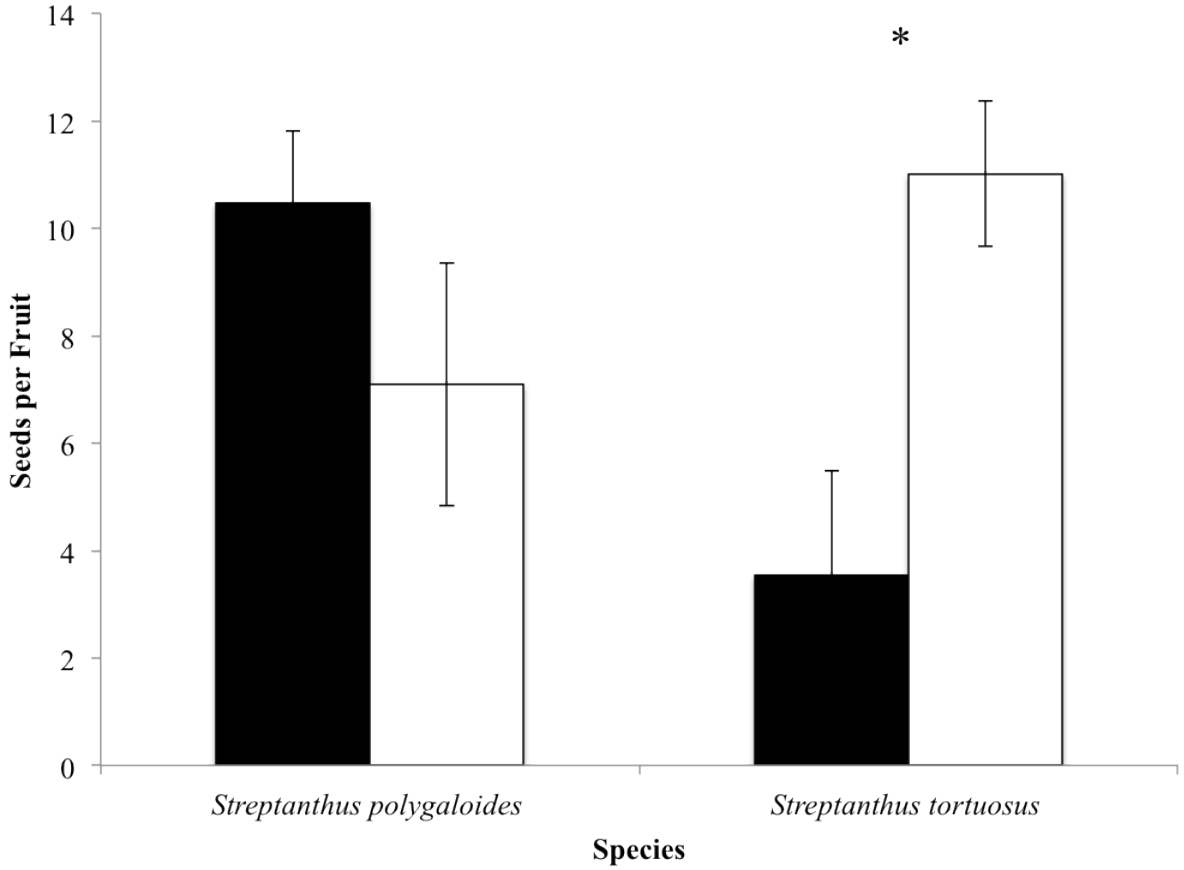

Figure 19. The number of seeds produced per fruit for *Streptanthus polygaloides* and *S. tortuosus* plants that were either grown in Ni-treated soils (black bars) or soils that were not Ni-treated (white bars). Bars represent lsmeans and associated standard errors. Asterisks indicate significant (i.e., adjusted *P*-value < 0.05) pairwise Tukey tests.

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