

# The role of selenium in protecting plants against prairie dog herbivory: implications for the evolution of selenium hyperaccumulation

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**Abstract** Some plants can hyperaccumulate the element selenium (Se) up to 10,000 mg Se kg<sup>-1</sup> dry weight. Hyperaccumulation has been hypothesized to defend against herbivory. In laboratory studies high Se levels protect plants from invertebrate herbivores and pathogens. However, field studies and mammalian herbivore studies that link Se accumulation to herbivory protection are lacking. In this study a combination of field surveys and manipulative field studies were carried out to determine whether plant Se accumulation in the field deters herbivory by black-tailed prairie dogs (*Cynomys ludovicianus*). The Se hyperaccumulator *Astragalus bisulcatus* (two-grooved milkvetch) occurs naturally on seleniferous soils in the Western USA, often on prairie dog colonies. Field surveys have shown that this Se hyperaccumulator is relatively abundant on some prairie dog colonies and suffers less herbivory than other forb species. This protection was likely owing to Se accumulation, as judged from subsequent manipulative field experiments. When given a choice between pairs of plants of the Se hyperaccumulator *Stanleya pinnata* (prince's plume) that were pretreated with or without Se, prairie dogs preferred to feed on the plants with low Se; the same results were obtained for the non-hyperaccumulator *Brassica juncea* (Indian mustard). Plants containing as little as 38 mg Se kg<sup>-1</sup> DW were protected from herbivory. Taken together these results shed light on the functional significance of Se hyperaccumulation and the possible selection

pressures driving its evolution. They also have implications for the use of plants in Se phytoremediation, or as Se-fortified crops.

**Keywords** *Astragalus bisulcatus* · Black-tailed prairie dog · *Stanleya pinnata*

## Introduction

While many plant species growing on soils with high concentrations of metals or metalloids have elevated tissue concentrations of those elements (Brooks 1987), some plant species hyperaccumulate these elements to levels several orders of magnitude higher than other plant species found on the same site (Baker and Brooks 1989). Over 400 species of plants have been reported to be hyperaccumulators of elements such as Ni, Zn, Cd, Cu, Co, Mn and Se (Reeves and Baker 2000). Selenium hyperaccumulators contain greater than 1000 mg Se kg<sup>-1</sup> dry weight (DW) and typically occur on seleniferous soils, such as those found in the Western USA (Beath et al. 1939a, b; Feist and Parker 2001). Some species of *Astragalus* and *Stanleya*, both native to the Western USA, accumulate upwards of 1000 mg Se kg<sup>-1</sup> DW from soils containing 4–10 mg Se kg<sup>-1</sup> DW (Shrift 1969; Feist and Parker 2001; Pickering et al. 2003).

Selenium is an essential trace element for mammals (Stadtman 1990), and Se hyperaccumulating plants may be a useful source of dietary Se (Ellis and Salt 2003; Freeman et al. 2006a). However, Se is toxic at higher levels, and ingestion of hyperaccumulators is responsible for a loss of livestock valued at \$330 million annually in the USA (Rosenfeld and Beath 1964; Wilber 1980). Selenium toxicity is thought to result from its chemical similarity to sulfur

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(S), leading to inadvertent uptake and replacement of S by Se in proteins and other redox-important S compounds (Stadtman 1990). It is unlikely that Se serves an essential role in higher plants, but elevated levels of Se are toxic to plants (Anderson 1993). As a result of its similarity to sulfate, selenate is incorporated into selenocysteine (SeCys) via the sulfate assimilation pathway. SeCys replaces cysteine in proteins, resulting in a loss of protein function; this process is in part responsible for Se toxicity (Stadtman 1990; Anderson 1993). Selenium hyperaccumulators have evolved a tolerance to Se by methylating SeCys into methylselenocysteine (MeSeCys), which is not easily incorporated into proteins (Brown and Shrift 1981).

Many hypotheses for the functional significance of metal and metalloid hyperaccumulation by plants have been proposed, including allelopathy, drought resistance and protection from both herbivores and pathogens (Reeves et al. 1981; Boyd and Martens 1992). There is considerable evidence for the latter, termed the elemental defense hypothesis. For example, high levels of Ni in plants have been shown to protect them from a variety of herbivores and pathogens (Boyd et al. 1994, 2002; Martens and Boyd 2002). Zinc and Cd can also protect plants from invertebrate herbivory (Pollard and Baker 1997; Jhee et al. 1999). Mounting evidence suggests that Se can protect plants from a variety of herbivores and pathogens, including rats, Lepidoptera larvae, aphids and fungal pathogens (Franke and Potter 1936; Vickerman and Trumble 1999; Bañuelos et al. 2002; Hanson et al. 2003, 2004; Freeman et al. 2006b). In further support of a defensive role, it appears that the Se in hyperaccumulators is concentrated in organs and tissues that are most susceptible to both herbivory and pathogens (Freeman et al. 2006a; Galeas et al. 2007). Selenium in hyperaccumulators is most concentrated in young leaves and reproductive tissues, and in trichomes and epidermal cell layers, all of which are the first to come into contact with attackers and often contain chemical defense compounds.

Ecologically relevant field studies examining the role of Se as an elemental defense in native hyperaccumulator environments are currently lacking. Boyd (2007) pointed to the lack of elemental defense studies regarding mammalian herbivores in his recent review of elemental defense literature. The study reported here expands on laboratory experiments, testing the ecological significance of Se hyperaccumulation in the field, and examines the possible role Se plays in protecting plants from mammalian herbivory. Specifically, this study explores the role Se may play in protecting plants from black-tailed prairie dog (*Cynomys ludovicianus*) herbivory. This small, burrowing mammal is one of the predominant herbivores in the natural habitat of Se hyperaccumulators in the Western United States. Prairie dogs live in large colonies and act as ecosystem engineers

(Jones et al. 1997), altering various aspects of their habitat, including plant community structure and species composition (Whicker and Detling 1988; Weltzin et al. 1997). Prairie dogs are voracious herbivores, both consuming and clipping vegetation. This facilitates predator avoidance by increasing visibility (Summers and Linder 1978; Uresk 1984; Hoogland 1995; Detling 1998). Since prairie dogs and Se hyperaccumulators are native to the same region, it is possible that prairie dog herbivory has acted as a selection pressure for the evolution of Se hyperaccumulation as an elemental defense. In this study several field surveys and manipulative field experiments were conducted to test the hypothesis that hyperaccumulated Se protects plants by deterring prairie dog herbivory.

## Materials and methods

### Field sites

Two field sites with similar characteristics were chosen to conduct experiments: Prairie Dog Meadow in South Fort Collins, Colorado (40°30.37N, 105°03.69W), and Pine Ridge Natural Area in South-West Fort Collins (40°32.70N, 105°07.87W). Both sites harbored the native Se hyperaccumulator *Astragalus bisulcatus* (two-grooved milkvetch, Fabaceae), which is an indicator of seleniferous soils. While Prairie Dog Meadow has a dense prairie dog population throughout the entire site, the prairie dog colony on Pine Ridge Natural Area occupies only about half of the area, making it particularly appropriate to study the effect of prairie dogs on the abundance of Se hyperaccumulators. The vegetation at both sites consisted of native grasses and forbs.

Surveys of herbivory damage to both Se hyperaccumulators and non-hyperaccumulators on prairie dog towns

### Survey I

At Prairie Dog Meadow, a 160-m-long transect was selected on the prairie dog colony containing the Se hyperaccumulator *A. bisulcatus*. The transect was divided into sixteen 100-m<sup>2</sup> (10 × 10 m) subplots. *A. bisulcatus* and the non-Se hyperaccumulator *Rumex crispus* (curly dock, Polygonaceae) were the two most abundant forbs on the colony. They are similar in size, but *R. crispus* has large simple leaves with long petioles while *A. bisulcatus* has much smaller, compound leaves growing from multiple stems. Probably because of this difference in morphology, prairie dogs clip *R. crispus* at the bases of petioles, and *A. bisulcatus* at the bases of stems. *Astragalus bisulcatus* and *R. crispus* are both perennials, and the average number of

petioles for *R. crispus* and stems for *A. bisulcatus* was initially the same ( $36.9 \pm 5.4$  and  $37.8 \pm 4.9$  per plant, respectively,  $n = 10$ ). Plant damage and herbivory by prairie dogs were compared for both species by measuring two parameters: the number of clippings per plant, and the percentage of clippings eaten. Plant damage was determined by counting the number of leaves or stems that were missing from a plant. The percentage of these clippings that were removed (i.e. not found on the ground next to a plant) was used as an estimate of prairie dog plant consumption. Prairie dogs were likely to have been responsible for the herbivory measured because they were abundant on the site and because of the type of damage suffered by the plants. On each of the 16 subplots two random plants, when available, of each species were randomly selected and analyzed for clipping, herbivory damage and Se concentration.

### Survey II

In July–August 2005 at the Pine Ridge Natural Area six plots were selected: three on and three off the prairie dog colony. On-colony plots were chosen where high prairie dog activity was observed; each plot was at least 30 m apart. Off-colony plots were selected by choosing areas closest in proximity to the on-colony plots with no prairie dog mounds or observed prairie dog activity. Both on-colony and off-colony, two of the three plots were 625 m<sup>2</sup> (25 × 25 m), and one was 400 m<sup>2</sup> (20 × 20 m). The size differences were to maximize the area and number of replications of plots as well as the consistency of prairie dog activity on-colony, and to have similar replications off-colony. Within each plot, 25-m<sup>2</sup> subplots were randomly selected, four in the large plots and two in the smaller plots; these were used to compare differences between plots. Density and percentage ground cover of each forb species were compared between sites on and off the prairie dog colony. Density was calculated by counting the number of plants from each species in each subplot. To measure ground cover, we placed a 0.1-m<sup>2</sup> Daubenmire plot every 2 m along the diagonal of each subplot, resulting in three measurements per subplot; we then estimated the percentage ground cover of each species (Daubenmire 1959). Both ground cover and density were compared by calculating the means of the subplots to obtain a mean for the plot; the mean of the three on-colony plots was then compared with the mean of the three off-colony plots. Prairie dog herbivory and consumption were estimated for two plants per species per subplot in the on-colony plots by counting the number of clipped stems and how many of those clippings were removed. Based on prairie dog abundance in the area, their foraging behavior and the type of damage observed on the plants, it is likely that the clipping and herbivory measured was due to prairie dogs. To further compare Se hyperaccumulator

abundance on and off the prairie dog colony, we correlated *A. bisulcatus* mean density and mean percentage ground cover per plot with mean prairie dog mound number per plot. The youngest mature leaves of plants analyzed for herbivory were used for Se analysis, as described below.

### Manipulative field studies of the effect of Se hyperaccumulation on prairie dog herbivory

#### *Twenty-four-hour Se feeding preference experiment*

To determine their feeding preference with respect to Se, prairie dogs in their natural habitat were given a choice between plants treated with or without Se. First, a 24-h study was performed using *Brassica juncea* (Indian mustard, Brassicaceae). *Brassica juncea* plants were used because they can accumulate fairly high levels of Se and do not occur naturally on the study site so the animals have not learned to avoid them based on appearance. Seeds (*B. juncea* Czern., accession no. 173874) were obtained from the North Central Regional Plant Introduction Station (Ames, Iowa). The plants were grown from seed in Premier Pro-Mix BX (Premier Horticulture, Quakertown, PA) potting soil in a growth room (12/12 h (light/dark), 24/20°C; 120 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic photon flux). After 2 weeks of growth, one half of the plants was watered three times a week with 40 μM Na<sub>2</sub>SeO<sub>4</sub> for 5 weeks while the other half was given water as a control. Leaf Se levels were determined as described below before plants were placed in the field.

To offer the prairie dogs a choice to feed on either plants with or without Se, a +Se *B. juncea* plant was placed 15 cm apart from a –Se plant on the prairie dog colony at Pine Ridge Natural Area in a high prairie dog density area, and herbivory was measured after 12 and 24 h ( $n = 30$  pairs of plants). The potted plants were placed in the field in holes so that the base of each plant stem was at ground level. Each pair of plants with or without Se was placed 20 m from another pair of plants on a densely populated prairie dog colony. Pairs of plants were the same size (approx. 25 cm tall), and there were no visible phenotypic differences between plants with or without Se. The number of leaves removed after 12 and 24 h was used as a measure of herbivory.

#### *Three-week Se feeding preference experiment*

A second choice feeding experiment was performed using the Se hyperaccumulator *Stanleya pinnata* plants treated with or without Se (prince's plume, Brassicaceae). *Stanleya pinnata* seeds were obtained from Western Native Seed (Coaldale, CO) and Plants of the Southwest (Sante Fe, NM). Plants were grown from seed in a growth room under

the same conditions as those described for *B. juncea*. After 5 weeks, half of the plants were treated with  $40 \mu\text{M}$   $\text{Na}_2\text{SeO}_4$  three times a week for 8 more weeks, while the other half were given water as a control. Pairs of plants treated with or without Se ( $n = 20$  pairs) were planted on a prairie dog colony at Prairie Dog Meadow approximately 20 m apart in order to give prairie dogs a choice to feed on *S. pinnata* plants with or without Se. Each plant with Se was placed approximately 15 cm from a control plant without Se. Plants were watered in the field twice a week to prevent desiccation. Initially, no size or phenotypic differences were visible between plants with or without Se; the plants were approximately 12 cm tall. After 3 weeks, the number of leaves removed was counted to estimate prairie dog herbivory. At this time, tissue Se levels were also determined, as described below.

#### Measurement of leaf Se concentration

Sampled leaves (youngest mature leaf) were rinsed with distilled water and dried at  $50^\circ\text{C}$  for 48 h. The samples were digested essentially as described by Zarcinas et al. (1987). One milliliter of concentrated nitric acid was added to 100 mg of plant material, followed by heating for 2 h at  $60^\circ\text{C}$ , then 6 h at  $130^\circ\text{C}$ . The samples were diluted with distilled water to 10 ml and analyzed for Se by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) according to Fassel (1978).

#### Data analyses

Statistical analyses were performed using the software package JMP-IN ver. 3.2.6 (SAS Institute Cary, NC). A paired  $t$  test was used to compare Se concentrations of leaves of *B. juncea* treated with or without Se during the manipulative field studies. An unpaired Student's  $t$  test was

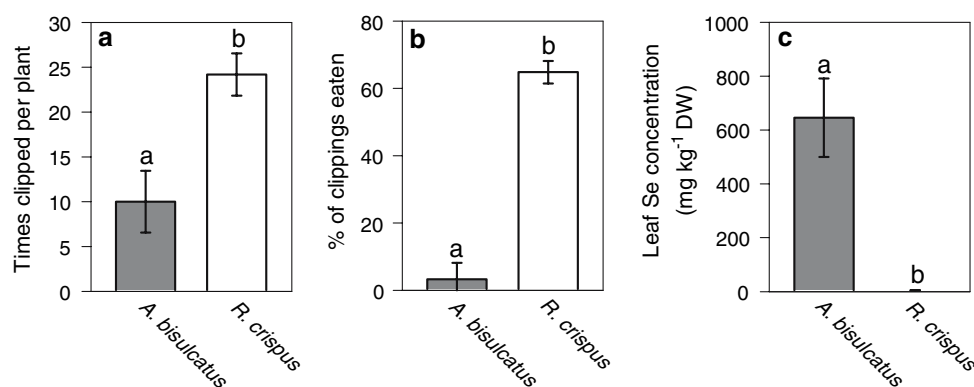
used to compare herbivory and clipping of *A. bisulcatus* and *R. crispus* at Prairie Dog Meadow, to compare the means of on- and off-colony plant density and percentage of ground cover at Pine Ridge and to compare Se leaf concentrations in plants. A Tukey–Kramer test was used to compare the herbivory and clippings between on-colony plant species at Pine Ridge. Pearson's  $R$  analysis was used to correlate prairie dog mound density to *A. bisulcatus* density and ground cover.

## Results

Surveys of herbivory damage to both Se hyperaccumulators and non-hyperaccumulators on prairie dog towns

#### Survey I

If Se acts as a deterrent to prairie dog herbivory, hyperaccumulators would be hypothesized to show less signs of herbivory in the field than a comparable non-hyperaccumulator species. To test this hypothesis, we conducted a comparative herbivory study on the predominant forbs on Prairie Dog Meadow, the Se hyperaccumulator *A. bisulcatus* and the non-Se hyperaccumulator *R. crispus*. *Astragalus bisulcatus* was clipped less often than *R. crispus* (Fig. 1a;  $t = -3.4$ ,  $n = 17$  for *A. bisulcatus*,  $n = 32$  for *R. crispus*,  $P = 0.0014$ ). In addition, *A. bisulcatus* appeared to be much less palatable, since on average only 3% of its clippings were apparently consumed compared with 65% for *R. crispus* (Fig. 1b;  $t = -10.3$ ,  $n = 17$  for *A. bisulcatus*,  $n = 32$  for *R. crispus*,  $P < 0.0001$ ). The *A. bisulcatus* at Prairie Dog Meadow contained  $645 \mu\text{g}$  Se  $\text{g}^{-1}$  DW, and *R. crispus* contained  $5 \mu\text{g}$  Se  $\text{g}^{-1}$  DW (Fig. 1c;  $t = 6.1$ ,  $n = 17$  for *A. bisulcatus*,  $n = 32$  for *R. crispus*,  $P < 0.0001$ ).



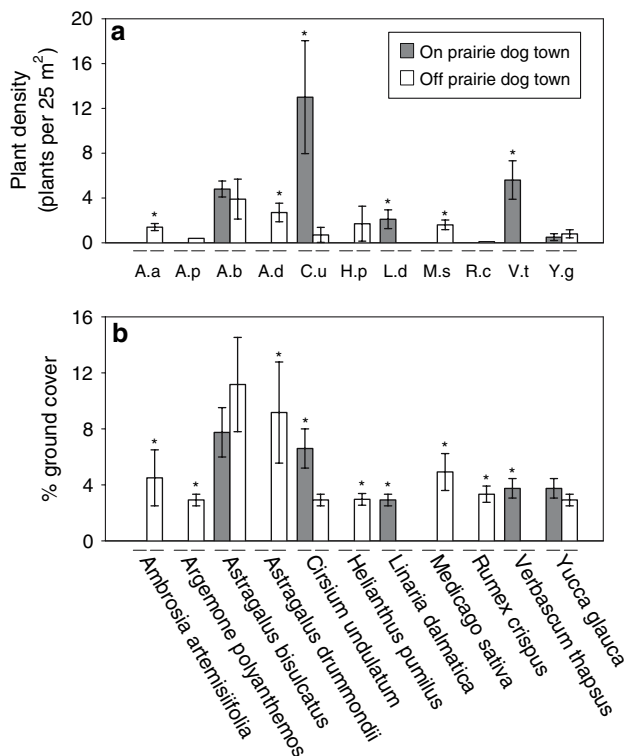
**Fig. 1** Survey comparing herbivory damage by prairie dogs at Prairie Dog Meadow. **a** Number of times the selenium (Se) hyperaccumulator *Astragalus bisulcatus* and non-hyperaccumulator *Rumex crispus* were

clipped, **b** percentage of those clippings removed. **c** Se concentration in leaf tissue of plants sampled. Values shown are means  $\pm$  SE. Different letters above bars represent significant differences ( $\alpha < 0.05$ )

## Survey II

If Se hyperaccumulators do suffer less herbivory on prairie dog colonies, it can be hypothesized that they will be relatively more abundant than other plant species on-colony rather than off-colony. To test this hypothesis, we conducted a survey comparing the density and ground cover of *A. bisulcatus* to those of other herbaceous non-grass species on and off a prairie dog colony at Pine Ridge Natural Area. In addition, all herbaceous non-grass species on-colony were assessed for prairie dog herbivory.

Of 11 non-grass plant species observed in the survey, all but two showed a significant difference in abundance between on-colony and off-colony (Fig. 2a, b). *Astragalus bisulcatus* was one of the two species for which no difference was found for mean percentage ground cover or density between on- and off-colony plots (Fig. 2a,  $t = -0.4$ ,  $n = 3$ ,  $P = 0.73$ ; Fig. 2b,  $t = 0.9$ ,  $n = 3$ ,  $P = 0.37$ ). There was no correlation between prairie dog mound density and *A. bisulcatus* density or percentage ground cover, but there were only six data points and this aspect will be explored further with more data collection.



**Fig. 2** Survey comparing plant species composition on and off a prairie dog colony at Pine Ridge. Density (a) and percentage ground cover (b) for all herbaceous non-grass species present on-colony (filled bars) and off-colony (open bars). Values shown are means  $\pm$  SE. An asterisk above a pair of bars represents a significant difference between on-colony and off-colony for that plant species ( $\alpha < 0.05$ )

Leaf Se concentration in *A. bisulcatus* was on average 3.7-fold higher on-colony than off-colony; this represents  $111 \mu\text{g Se g}^{-1}$  DW compared to  $39 \mu\text{g Se g}^{-1}$  DW, although the difference is not significant using an alpha of 0.05 ( $t = -1.9$ ,  $n = 8$  *A. bisulcatus* off-colony;  $n = 26$  *A. bisulcatus* on-colony;  $P = 0.07$ ). While both the on- and off-colony *A. bisulcatus* Se concentrations were significantly higher than those of the other species measured at the Pine Ridge Natural Area, which were both less than  $4 \mu\text{g Se g}^{-1}$  DW ( $t = 7.9$ ,  $n = 34$  *A. bisulcatus*;  $n = 98$  other species;  $P < 0.0001$ ), they were substantially lower than the  $645 \mu\text{g Se g}^{-1}$  DW found in *A. bisulcatus* growing at Prairie Dog Meadow ( $t = 5.2$ ,  $n = 17$  *A. bisulcatus* at Prairie dog Meadow,  $n = 34$  *A. bisulcatus* Pine Ridge;  $P < 0.0001$ ).

Observations suggested that *A. bisulcatus* was relatively abundant on the prairie dog colony at Pine Ridge Natural Area, and it was often observed growing in close proximity to active prairie dog mounds (Fig. 3a). In addition, *A. bisulcatus* was generally bigger and had more stems to clip than other species on the colony. However, it was not clipped significantly more often than any other species (data not shown). When compared with other species occurring on-colony, *A. bisulcatus* clippings were removed less than clippings from other species on the site, except *Linaria dalmatia*, which had similar results to *A. bisulcatus*, suggesting that the hyperaccumulators were eaten relatively less by the prairie dogs (Fig. 3b, c).

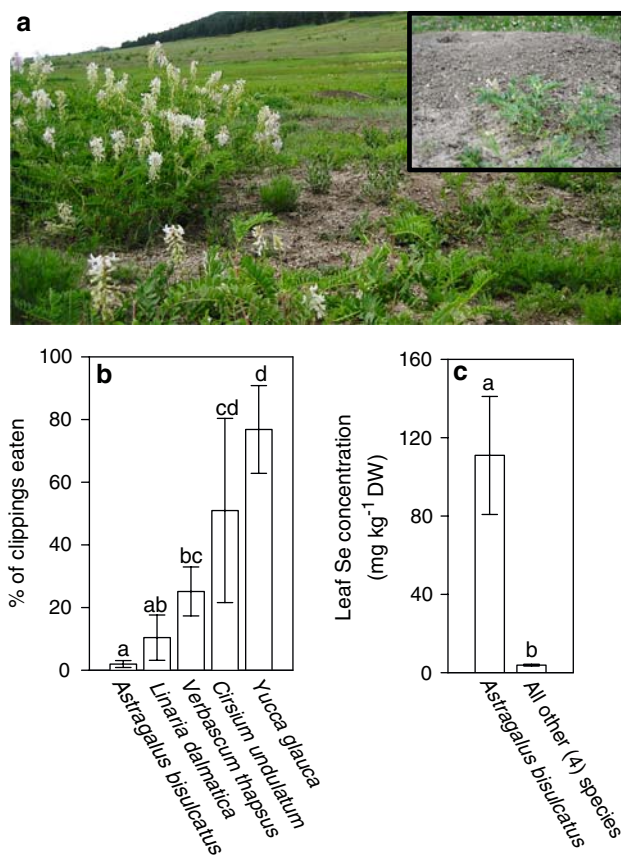
#### Manipulative field studies of the effect of Se hyperaccumulation on prairie dog herbivory

##### Twenty-four-hour Se feeding preference experiment

To determine under more controlled conditions whether plant tissue Se acts as a deterrent for prairie dogs, we performed two short-term manipulative field studies in which prairie dogs were given a choice between plants of the same species pretreated with or without Se. In a choice study over a 24-h period using the non-hyperaccumulator *B. juncea*, prairie dogs could distinguish between plants with and without Se. Prairie dogs preferentially fed on plants that contained significantly less Se (Fig. 4c;  $t = -5.7$ ,  $n = 30$  pairs,  $P < 0.0001$ ), avoiding plants with high Se levels, both after the first 12 h (Fig. 4a;  $t = -2.6$ ,  $n = 30$  pairs,  $P = 0.015$ ) and again after 24 h (Fig. 4b;  $t = -3.8$ ,  $n = 30$  pairs,  $P = 0.0005$ ).

##### Three-week Se feeding preference experiment

To further test whether Se protects hyperaccumulators from prairie dog herbivory, follow-up choice feeding studies were done with *S. pinnata*. Initially, the experiments were performed over 24 h on the Pine Ridge Natural Area, as



**Fig. 3** Survey comparing herbivory damage by prairie dogs at Pine Ridge. **a** *Astragalus bisulcatus* thriving on the prairie dog colony at Pine Ridge and growing directly on an active prairie dog mound (*inset*). **b** Percentage of clippings removed from all five herbaceous non-grass species on a prairie dog colony. **c** Leaf Se concentration in Se hyperaccumulator *A. bisulcatus*, and the average of all non-Se hyperaccumulating species. Values shown are means  $\pm$  SE. Different letters above bars represent significant differences ( $\alpha < 0.05$ )

described for *B. juncea*. Of the 30 pairs of *S. pinnata* pretreated with or without Se that were offered, only three pairs were sampled; in all cases these were plants pretreated without Se (data not shown). When pairs of *A. bisulcatus* treated with or without Se were offered at Pine Ridge Natural Area in a 24-h experiment, none of the plants were even

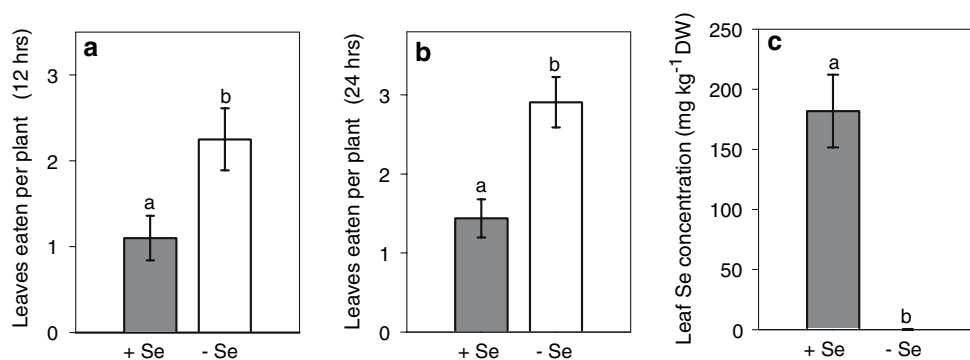
sampled. Given that *A. bisulcatus* and *S. pinnata* both occur naturally in the Pine Ridge Natural Area, it is possible that prairie dogs have learned to avoid these hyperaccumulators based on their appearance. Therefore, further experiments were done with *S. pinnata* on Prairie Dog Meadow, where *S. pinnata* did not occur naturally. The experiment was also performed over a longer time period, to give more time for herbivory. Prairie dogs preferred to feed on *S. pinnata* plants with low Se significantly more than on plants containing elevated levels of Se (Fig. 5a;  $t = 4.8$ ,  $n = 20$  pairs,  $P < 0.0001$ ). Mean plant Se concentration was  $3.0 \mu\text{g Se g}^{-1}$  DW for low Se and  $37.4 \mu\text{g Se g}^{-1}$  DW for high Se.

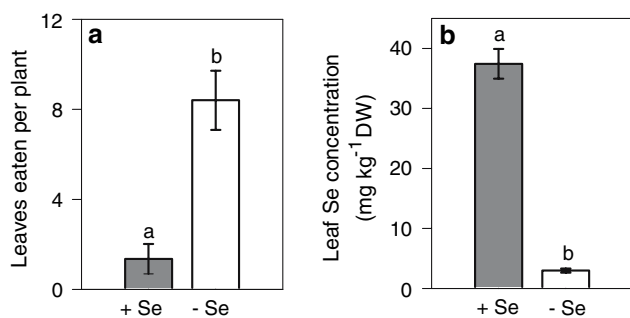
## Discussion

Results from this study support a protective function for hyperaccumulated Se against herbivory by prairie dogs in their native habitat. Field surveys indicated that Se hyperaccumulators were abundant on densely populated prairie dog colonies and suffered little herbivory compared to other non-grass herbaceous species on the site. Manipulative field studies showed that prairie dogs preferred to feed on plants without Se when given a choice between high or low Se plants of the same species, suggesting that accumulated Se deterred prairie dog herbivory.

An initial comparative herbivory survey indicated that the Se hyperaccumulator *A. bisulcatus* suffered less prairie dog herbivory than the non-Se hyperaccumulator *R. crispus*, the other predominant dicotyledon species on the Prairie Dog Meadow site. While plants of both species had the same average number of available leaves or stems to clip when undamaged, our survey revealed that *A. bisulcatus* was clipped and eaten less often than *R. crispus*. During another survey at Pine Ridge Natural Area, *A. bisulcatus* was one of only five non-grass species that were found on-colony. Of the four other species, three (*Yucca glauca*, *Cirsium undulatum* and *Verbascum thapsus*) have mechanical defenses that may protect them from prairie dog herbivory, and the fourth plant species, *Linaria dalmatica*, is rarely

**Fig. 4** Prairie dog choice feeding experiment using *Brassica juncea* plants treated with (+Se) or without Se (-Se). **a** Number of leaves removed after 12 h in the field. **b** Number of leaves removed after 24 h in the field. **c** Leaf Se concentration. Shown are means  $\pm$  SE. Letters above bars represent significant differences ( $\alpha < 0.05$ )





**Fig. 5** Prairie dog choice feeding experiment using the Se hyperaccumulator *Stanleya pinnata* plants treated with (+Se) or without Se (–Se). **a** Number of leaves removed after 3 weeks in the field. **b** Leaf Se concentration. Shown are means  $\pm$  SE. Different letters above bars represent significant differences ( $\alpha < 0.05$ )

consumed by wildlife or grazing animals; it contains alkaloids (Polunin 1969) that may also protect it from prairie dog herbivory. Among the five non-grass species on colony, *A. bisulcatus* appeared to suffer the least prairie dog herbivory.

The apparent protective effect of Se in these *A. bisulcatus* plants is especially interesting because the Se levels were relatively low  $\sim 110 \text{ mg kg}^{-1} \text{ DW}$  (Fig. 3c). Levels of 4000–7000  $\text{mg kg}^{-1} \text{ DW}$  are often seen in *A. bisulcatus* in the field (Galeas et al. 2007). Intriguingly, on this site, the *A. bisulcatus* Se concentration was on average threefold higher on-colony than off-colony, although this difference was not significantly different. Further studies may indicate that Se accumulation is upregulated in response to herbivory pressure. It is also possible that the plant Se concentrations simply reflected genetic variation or increased soil Se concentration or availability. The soil Se concentration was below a detection level of  $0.5 \mu\text{g Se g}^{-1} \text{ DW}$  (data not shown). However, in an earlier study on a nearby plot where soil Se levels were higher, no correlation was found between Se concentration in the soil and in *A. bisulcatus* leaves (Galeas et al. 2007).

Because the tissue Se in *A. bisulcatus* appears to provide protection against prairie dog herbivory, it is possible that the competitive advantage *A. bisulcatus* has over other species would lead to increases in *A. bisulcatus* density on prairie dog towns. There was no significant correlation between *A. bisulcatus* abundance and prairie dog mound density, but it would be interesting to explore this possibility further.

There were nine non-grass herbaceous species off-colony on the Pine Ridge Natural Area compared to five species on-colony. This result differs from earlier, more extensive studies where forb species richness was higher on rather than off prairie dog colonies (Detling 1998; Fahnestock et al. 2003). It may be interesting to survey more seleniferous sites in order to test whether prairie dog colonies affect species richness differently on Se soils than on soils without Se.

As mentioned, prairie dogs preferred to feed on plants without Se when given a choice between high or low Se plants from the same species. Similar results were found for the non-hyperaccumulator, *B. juncea* and the Se hyperaccumulator, *S. pinnata*. However, the Se concentrations found in these plants were not as high as those typically found in hyperaccumulators. Since the form of Se in hyperaccumulators is MeSeCys (Shrift and Virupaksha 1965; Dunnill and Fowden 1967; Freeman et al. 2006b) and non-hyperaccumulating species mainly accumulate selenate (de Souza et al. 1998; Pilon-Smits et al. 1998, 1999), this study suggests that both these inorganic and organic forms of Se provide protection from prairie dog herbivory. The observation that the high Se plants were not left untouched suggests that after tasting these plants, the prairie dogs preferred to continue feeding on plants with lower Se. Apart from a possible taste associated with Se accumulation, it is likely that the prairie dogs sensed volatile plant selenocompounds, which are produced by both non-hyperaccumulators (dimethylselenide) and hyperaccumulators (dimethyldiselenide) (de Souza et al. 1998). Elevated metal concentrations have been isolated as the causal protective agent against herbivory (Behmer et al. 2005), and volatile selenocompounds have a distinct smell that can be readily detected even by humans (Evans et al. 1968). Since these choice studies offered plants of the same species and phenotype, which were watered with the same solution except for different Se concentrations, these results strongly suggest that the prairie dogs could detect – and avoid – plants with Se levels as low as  $38 \text{ mg Se g}^{-1} \text{ DW}$ .

The finding that Se accumulation protects plants from prairie dog herbivory has several implications. Importantly, it sheds light on the evolution and possible functional significance of hyperaccumulation. This study provides support for the elemental defense hypothesis, which proposes that metal or metalloid hyperaccumulation protects plants from herbivores or pathogens (Boyd and Martens 1992). The finding that Se accumulation protects plants against prairie dog herbivory makes it feasible that herbivory by small mammals, such as the prairie dog or its evolutionary ancestor, has contributed in driving the evolution of Se hyperaccumulation in areas where these herbivores are abundant, such as on the short grass steppe. Herbivory pressure from other animals may have contributed in driving the evolution of Se hyperaccumulation in different habitats. It has been shown that other mammals are also deterred from feeding on Se-containing plants, as reported for rats (Franke and Potter 1936), and Se has been shown to deter feeding by many invertebrate species (for a review see Quinn et al. 2007).

The concentrations of Se in plant tissues that were shown here to be effective in deterring prairie dog herbivory

ranged from 38 to 650 mg kg<sup>-1</sup> DW. Thus, even at levels that are one to two orders of magnitude lower than those typically found in native hyperaccumulators, Se appears to confer herbivory protection. These results suggest that prairie dogs have a low Se concentration deterrence threshold; it is possible that the more elevated levels of Se found in hyperaccumulators in the field serve to protect against other, more Se-tolerant or -insensitive herbivores or pathogens, or Se hyperaccumulation may serve another or even multiple roles that have not yet been identified. Also, if tissue levels as low as 38 mg kg<sup>-1</sup> DW offer protection, and the form of Se found in non-hyperaccumulators effectively protects plants, these findings have implications for the cultivation of Se-fortified crop species and plants used in phytoremediation. Recently, there has been an increasing interest in using plants as a dietary source of anticarcinogenic selenocompounds and to help clean up Se pollution (Clark et al. 1996; Reid et al. 2002; Ellis and Salt 2003; Pilon-Smits and Freeman 2006). These results suggest that low levels of accumulated Se protect *B. juncea*, *S. pinnata* and *A. bisulcatus* from prairie dogs; it is possible that accumulated Se will deter other herbivores from various plant species and act as a pesticide. Selenium added to irrigation water and accumulated by crops would be a valuable pesticide because it has the unique characteristic of being both toxic and beneficial. If Se-fortified crops are harvested, this prevents the amount of unwanted Se entering ecosystems through senescence and provides a valuable nutrient-enriched crop to supplement Se-deficient diets. In addition, the accumulation of Se by crops may provide medical benefits through the ingestion by livestock or humans who live in low-Se areas.

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