

Chenopod salt bladders deter insect herbivores

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Abstract Trichomes on leaves and stems of certain chenopods (Chenopodiaceae) are modified with a greatly enlarged apical cell (a salt bladder), containing a huge central vacuole. These structures may aid in the extreme salt tolerance of many species by concentrating salts in the vacuole. Bladders eventually burst, covering the leaf in residue of bladder membranes and solid precipitates. The presence of this system in non-halophytic species suggests additional functions. I tested the novel hypothesis that these bladders have a defensive function against insect herbivores using choice, no choice, and field tests. Generalist insect herbivores preferred to feed on leaves without salt bladders in choice tests. In no choice tests, herbivores consumed less leaf matter with bladders. In a field test, leaves from which I had removed bladders suffered greater herbivory than adjacent leaves with bladders. Solutions containing bladders added to otherwise preferred leaves deterred herbivores, suggesting a water-soluble chemical component to the defense. This bladder system has a defensive function in at least four genera of chenopods. Salt bladders may be a structural defense, like spines or domatia, but also have a chemical defense component.

Keywords Plant–herbivore interaction · Salt bladders · Salt hairs · Vesicular hairs · Chenopodiaceae · *Chenopodium* · *Atriplex*

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Introduction

Many physical structures on plants provide protection from herbivores, either directly (e.g., spines; Cooper and Ginnett 1998) or indirectly through mutualisms (e.g., ant domatia; Vasconcelos 1991) or provisioning of carnivores (e.g., carrion entrapment, Krimmel and Pearse 2013). Chemically, plant tissues contain organic (e.g., tannins) or inorganic (e.g., Ni, Martens and Boyd 1994) compounds that reduce palatability to herbivores. Leaf structures can provide a variety of physiological benefits as well (e.g., reflecting light to reduce leaf temperature or reducing boundary flow). Most structures have probably been selected upon for a variety of functions, for instance, trichomes can serve all of the above functions.

Many families of plants have convergently evolved salt glands, which allow the plant to excrete various ions against a concentration gradient (Lipshitz and Waisel 1982; Schirmer and Breckle 1982). Unlike salt glands, which excrete salt immediately, epidermal bladder cells (EBCs) sequester salts in a large central vacuole and are found in several plant families now in halophytic and non-halophytic environments (Lipshitz and Waisel 1982; Schirmer and Breckle 1982). In the well-studied iceplant, *Mesembryanthemum crystallinum*, and other Aizoaceae, these EBCs are greatly enlarged cells on the surface of the leaf (Adams et al. 1998; Opel 2005). Bladders of the chenopods (of the Chenopodiaceae–Amaranthaceae alliance; sensu Fuentes-Bazan et al. 2012b) are modified trichomes and are usually separated from the leaf by one to four thin stalk cells (Schirmer and Breckle 1982; Fig. 1a). These structures have been referred to as “salt hairs” (e.g., Karimi and Ungar 1989), “inflated unicellular trichomes” (e.g., Chu et al. 1991), or “vesicular hairs” (e.g., Black 1954; Black 1958; Mozafar and Goodin 1970; Fuentes-Bazan

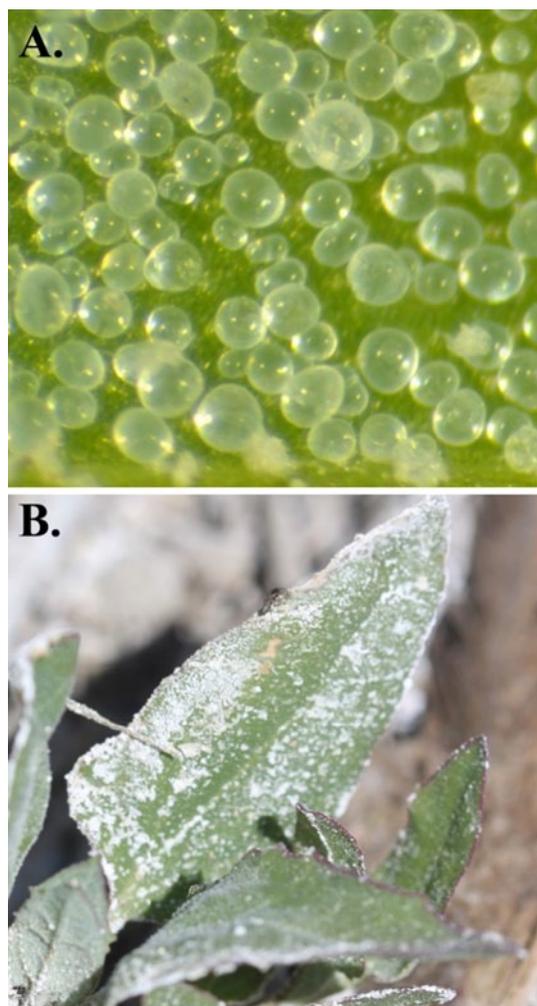


Fig. 1 **a** Bladders on the underside of a young *Atriplex prostrata* leaf. **b** The same species with post-rupture precipitate on leaves. Both photos from the UC Davis McLaughlin Reserve, Lake County, CA, USA

et al. 2012b). Similar to salt glands, these structures may be important in salt regulation in many species (Schirmer and Breckle 1982; Adams et al. 1998), though Waisel (1970) suggests that the total amount of Na^+ sequestered by bladders is too low to make an appreciable impact on the plant's sodium balance. In certain saltbushes, *Atriplex* spp., vacuoles in bladder cells contain almost fully saturated solutions of NaCl , (~5 M: Mozafar and Goodin 1970; Schirmer and Breckle 1982). Interestingly, these structures concentrate ions (e.g., sodium, chloride, potassium, etc.) even under low or no salinity stress and in non-halophytic species (Mozafar and Goodin 1970; Schirmer and Breckle 1982; Orsini et al. 2011), a potentially costly activity. Bladders eventually collapse, leaving a film of crystallized solids and dried bladder membranes on the surface of the leaf (Fig. 1b).

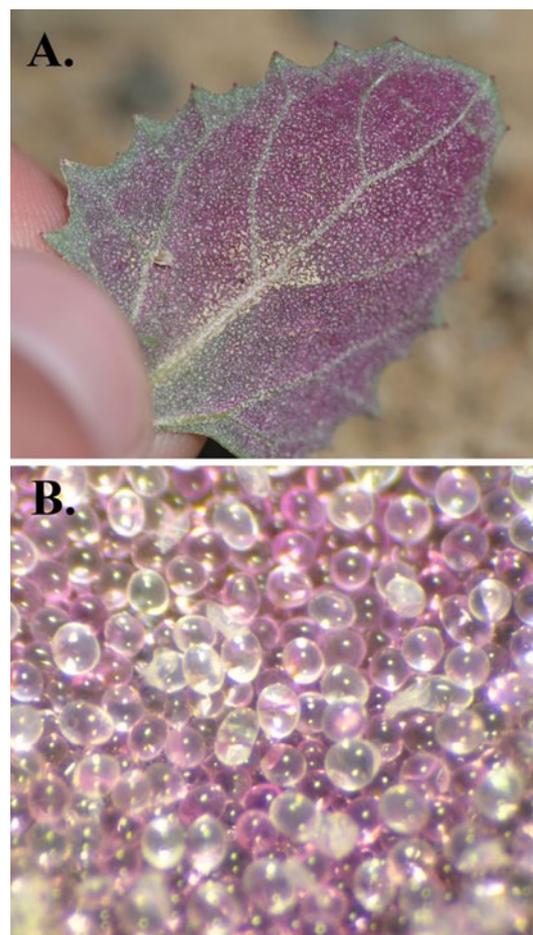


Fig. 2 **a** Purplish coloration on the underside of an *Atriplex rosea* leaf, likely caused by betalains. **b** No part of the leaf itself is colored, only specific bladders. Note the margins and stems are whitish green; they have low bladder density compared to the leaf surface. McLaughlin Reserve, Lake County, CA, USA

These bladders are well-studied physiologically. In *Atriplex* spp. and *M. crystallinum*, the number and size of the salt bladders may be positively correlated with salinity (Black 1958; Karimi and Ungar 1989; Adams et al. 1998; but see Schirmer and Breckle 1982). In quinoa (*Chenopodium quinoa*), the bladders functioned as salt reservoirs at low salinities; increasing salinity beyond a certain point caused leaves to accumulate Na^+ (Orsini et al. 2011). In certain *Atriplex* spp., the bladders are continuously formed during ontogeny (Black 1954), whereas in *C. quinoa* (and herbaceous *Atriplex*, *Chenopodium*, certain *Chenopodium*, and *Oxybassis* spp.) they are formed only early in leaf development and collapse within a few weeks after leaf emergence (personal observation). Schirmer and Breckle (1982) note that certain *Chenopodium* species with bladders are completely salt-intolerant, evidence suggestive of alternate functions.

Bladders may also reflect light, reducing leaf temperature in heat-stressed environments, like other leaf-hairs

(e.g., Ehleringer and Mooney 1978). In nearly all species of the family with bladders, the bladders and residue after rupture are densest on the underside of the leaves, suggesting reflectance of sunlight is not a primary function for most species (personal observation; predominance on undersides noted in Baldwin et al. 2012). The presence of bladders may increase the boundary layer and reduce transpiration (J. Richards, personal communication), or may allow the uptake and excretion of heavy metals (postulated in Schirmer and Breckle 1982, but not yet tested to the best of my knowledge). Though the bladders absorb water from humidity or rain, they do not seem to confer drought resistance through water storage or movement of water into leaf tissue (Black 1954, Mozafar and Goodin 1970).

Correlational evidence suggests that bladders may serve a defensive function. Proteins identified in bladders of iceplant are known from other plants to have antiviral and antifungal properties (Jou et al. 2007). Calcium oxalate crystals may be concentrated in the bladders and are important in K^+ retention under salt stress (Jou et al. 2007). In several non-bladdered species, these crystals deter vertebrate and invertebrate herbivory (Ward et al. 1997), and formation of oxalate crystals can be induced by herbivory (Molano-Flores 2001). Betalains, a class of secondary metabolites found in the Caryophyllales, are localized in bladders of chenopods giving them reddish-purple coloration (Fig. 2a, b; Adams et al. 1998; Adolf et al. 2013). Betalain levels are negatively correlated with herbivory in *Amaranthus*, consistent with a defensive function (Niveyro et al. 2012).

The only direct evidence for a defensive function of the bladder system comes from studies of rodents and *Atriplex*. A single species of kangaroo rat, *Dipodomys microps*, in western North America has uniquely flat incisors used to scrape off the salt depositions (post-bladder rupture) on the leaf surface of saltbush in order to access inner tissue; all other kangaroo rat species lack this adaptation and do not consume saltbush foliage (Kenagy 1972, 1973). In a remarkable case of convergent evolution, two species of distantly-related rodents in Africa and South America have evolved similar dental morphology and consume *Atriplex* foliage (Mares et al. 1997). Other than this example, defensive functions of salt bladders or post-rupture residue are unknown. What physiological and ecological functions salt bladders and post-rupture residue serve in non-halophytic environments is similarly unknown.

The presence of bladders on many species in areas without obvious heat or salt stress is suggestive of additional functions. Their location on leaf and stem surfaces makes bladders the first tissue contacted by chewing insect herbivores. I hypothesize that bladders serve a defensive function in many chenopods. *Blitum*, *Chenopodium*, *Chenopodium*, and many *Atriplex* spp. in northern California

suffer heavy herbivory from the highly polyphagous adult spotted cucumber beetle, *Diabrotica undecimpunctata* (Coleoptera: Chrysomelidae) and armyworms, *Spodoptera exigua* (Lepidoptera: Noctuidae). They are attacked by other herbivores including an uncommon leaf miner, *Monoxia angularis* (Coleoptera: Chrysomelidae), which may be a specialist, and an uncommon and very seasonal butterfly, *Brephidium exile* (Lepidoptera: Lycaenidae), which is a specialist. All herbivores found in a study of quinoa in Peru were generalists (Yábar et al. 2002). These data and the observation of few other herbivores despite intense searching suggest that generalist herbivores may be important consumers of chenopods in northern California. I therefore performed a series of choice and no choice tests to test the hypothesis that the salt bladders, post-rupture residue from bladders, and chemicals within bladders of several genera of chenopods deter generalist herbivores. I also performed a similar test using solutions of NaCl at ecologically relevant concentrations, a likely deterrent compound in certain chenopod bladders.

Materials and methods

Choice tests

I performed 24 choice tests involving chenopod leaves and three generalist herbivores: the woolly bear, *Platypreria virginialis*, the tobacco budworm, *Heliothis virescens*, and the spotted cucumber beetle *Diabrotica undecimpunctata*. The former eats small quantities of *Blitum californicum* (Chenopodiaceae) in coastal California; the latter two consume chenopods of several genera in the central valley of California (personal observation). To test whether salt bladders deterred these herbivores, I gently removed bladders on one side of the midvein of an excised leaf. The stalk cells below the bladder readily break and allow removal of bladders with a paintbrush without damage to the leaf surface. After bladder rupture, they are easily removable with a soft sponge and distilled water, after which the leaves were dried before testing. Neither removal of intact or collapsed bladders removed 100 %; the amount removed in each test was ~75–90 %. I alternated which side I removed bladders from on each leaf to control for any directional bias of the herbivores. I then placed the leaves ventral side up in 59-mL condiment cups (Dart Industries, Los Angeles, CA, USA). I placed leaves ventral side up for two reasons: leaves were generally more stable as the leaf was often angled up at the midvein and it presented the herbivore with the starkest contrast, as bladders are densest on the ventral side. I then added the herbivores, starved overnight, to the condiment cups. I performed tests in a laboratory at UC Davis. Numbers of replicates varied with daily

Table 1 Bladder-removal choice tests

No.	Genus	Species	Annual/ perennial	Plant origin	Native range	Plant stage	Bladder stage	Herbivore	Bladders	No bladders	No choice	<i>p</i>
1	<i>Atriplex</i>	<i>argentea</i>	Perennial	USDA, PARL 110 ^b	North America	Preflowering	Collapsed	<i>Diabrotica</i>	0	8	17	0.0039 ^c
2		<i>hortensis</i>	Annual	USDA, PI 310383 ^a	Europe	Preflowering	Intact	<i>Diabrotica</i>	10	15	5	0.2122
3		<i>lentiformis</i>	Perennial	UCD arboretum: Mojave Desert origin	North America	Preflowering, leaves older	Collapsed	<i>Diabrotica</i>	4	20	16	0.0008
4		<i>prostrata</i>	Annual	Davis, CA, wild plants	Europe	Preflowering	Not noted	<i>Diabrotica</i>	4	16	12	0.0059
5		<i>prostrata</i>	Annual	USDA, W6 30007 ^a	Europe	Preflowering	Collapsed	<i>Diabrotica</i>	6	14	10	0.0577
6		<i>vesicaria</i>	Perennial	UCD arboretum: Ballarat Seed Bank, AUS ^a	Australia	Preflowering	Intact	<i>Diabrotica</i>	3	21	1	0.0001
7	<i>Blitum</i>	<i>californicum</i>	Perennial ^b	Bodega Bay, wild plants	North America	Preflowering	Intact	<i>Platyrepia</i>	6	16	8	0.0262 ^{d,e,f}
8	<i>Chenopodium</i>	<i>album/berlandieri</i>	Annual	Sacramento County, wild plants ^a	Europe/North America	Preflowering	Collapsed	<i>Diabrotica</i>	3	11	7	0.0287
9		<i>album/berlandieri</i>	Annual	Sacramento County, wild plants ^a	Europe/North America	Preflowering	Intact	<i>Diabrotica</i>	12	12	6	0.5806
10		<i>album</i>	Annual	USDA, PI 605700 ^a	Europe	Preflowering	Intact	<i>Diabrotica</i>	8	21	1	0.0121
11		<i>album</i>	Annual	USDA, PI 658736 ^a	Europe	Preflowering	Intact	<i>Diabrotica</i>	5	15	10	0.0207
12		<i>berlandieri</i> spp. <i>nut-talitanum</i>	Annual	USDA, PI 568155 ^a	North America	Preflowering	Intact	<i>Diabrotica</i>	13	16	1	0.3555
13		<i>berlandieri</i> var. <i>bushianum</i>	Annual	USDA, PI 608030 ^a	North America	Preflowering	Intact	<i>Diabrotica</i>	4	19	7	0.0013
14		<i>formosanum</i>	Annual	USDA, PI 433378 ^a	Asia	Preflowering	Intact	<i>Diabrotica</i>	8	14	8	0.1431
15		<i>fremontii</i>	Annual	USDA, PI 666302 ^a	North America	Preflowering	Intact	<i>Diabrotica</i>	3	10	17	0.0461
16		<i>giganteum</i>	Annual	USDA, PI 667180 ^a	Asia	Preflowering	Intact	<i>Diabrotica</i>	5	19	1	0.0033
17		<i>neomexicanum</i>	Annual	USDA, PI 268143 ^a	North America	Preflowering	Intact	<i>Diabrotica</i>	7	17	1	0.032
18		<i>neomexicanum</i>	Annual	USDA, PI 268143 ^a	North America	Flowering	Collapsed	<i>Diabrotica</i>	4	13	13	0.0245
19		<i>pallidicaule</i>	Annual	USDA, PI 478406 ^a	South America	Preflowering	Intact	<i>Diabrotica</i>	5	12	8	0.0717
20		<i>quinoa</i>	Annual	Safeway organic Quinoa ^a	South America	Preflowering	Intact	<i>Heliothis</i>	2	9	1	0.0327
21		<i>Quinoa</i>	Annual	Safeway organic Quinoa ^a	South America	Preflowering	Intact	<i>Diabrotica</i>	7	26	2	0.0006
22		<i>spinescens</i>	Perennial	UCD arboretum: Australian origin	Australia	Preflowering, leaves older	Collapsed	<i>Diabrotica</i>	3	20	6	0.0002
23	<i>Oxybasis</i>	<i>strictum</i>	Annual	USDA, PI 666324 ^a	North America	Preflowering	Intact	<i>Diabrotica</i>	10	14	6	0.2706
24		<i>glauca</i>	Annual	Davis, CA, wild plants ^a	Asia	Preflowering	Intact	<i>Diabrotica</i>	5	18	7	0.0053

Plant origin refers to the specific strain in question, *USDA* numbers refer to accession numbers in the USDA GRIN database. *Bladder stage* refers to whether the majority of bladders were intact or ruptured on the tested leaves. *Bladders* refers to number of herbivores which ate more leaf area on the side of the midvein with either intact bladders or unremoved residue. *No bladders* refers to the number of herbivores which ate more leaf area on the side of the midvein with either removed bladders or removed residue. *No choice* is the number of herbivores which ate no or equal quantities of leaf matter. *p* values are calculated from a two-tailed binomial test assuming equal likelihood of consuming either option

^a From seed

^b Grows from a perennial rootstock; all stems and leaves die back each year

^c Run overnight because plant is not a readily accepted foodplant

^d Two whole leaves used, one with bladders intact, other with bladders removed

^e Performed at the UC Davis Bodega Marine Laboratory, Sonoma County, CA, USA

^f Test conducted in a 15-cm-diameter Petri dish

availability of insects and plants. Any departures from this protocol are noted in Table 1.

I scored caterpillars as preferring a treatment if they consumed more leaf tissue of that side of the leaf. If consumption of the two treatments was near equality and could not be ascertained by eye, no preference was recorded. I ran all trials for 3–5 h, except where noted in Table 1.

I additionally performed several other choice tests. As woolly bears do not feed readily on *B. californicum*, I removed bladders from this plant and dipped wetted leaflets of two preferred host plants (lupine *Lupinus arboreus*, and poison hemlock *Conium maculatum*; Karban et al. 2010) in these bladders, leaving a film of bladders on the leaf surface. I then placed these next to wetted control leaflets, alternating right–left, in 15-cm Petri dishes and added woolly bears. I recorded their choices as above after 3–4 h.

To examine whether the deterrent properties of *B. californicum* were physical or chemical, I dissolved dried salt bladders in deionized water (0.045 g/ml H₂O) and wetted poison hemlock leaves with this and with control deionized water, and conducted the test with woolly bears following the protocols outlined above. I examined the same question in *Chenopodium spinescens* leaves by soaking 10 g of post-bladder-rupture leaves in 50 mL deionized water for 24 h, making a solution with which I coated bladder-removed leaves. As a control, I coated other bladder-removed leaves with deionized water and let both groups dry for 10 min before performing a choice test with locally-collected, starved cucumber beetles.

To examine whether sodium chloride, at ecologically relevant bladder concentrations, was a deterrent, I halved *A. prostrata* leaves, cutting out the midvein, and sprayed one-half of the leaf with 3 M NaCl. This represents a medium concentration for *A. halimus* (Mozafar and Goodin 1970). I sprayed the other half with deionized water and followed the above protocols. For the tests involving solutions and bladder additions, small amounts of liquid and bladders were used. As bladder mass can be 21 % of the total leaf weight in *Atriplex* (Osmond et al. 1969) or >25 % in *Chenopodium* (unpublished data), the small amount of spray and bladders used (surely <10 % total leaf weight) in these trials were certainly a conservative amount, though I did not quantify them exactly.

No choice tests

In the laboratory, I presented intact or bladder-removed leaves of *Chenopodium quinoa* to cucumber beetles (N1). I did the same with leaf punches (13 mm diameter) of both *A. rosea* (residue removed) collected from the McLaughlin reserve (N2), *Atriplex prostrata* (bladders removed) from both Davis and the McLaughlin reserve (N3a, b, respectively) and *A. lentiformis* from the UC Davis arboretum

(N4). All trials were run for 24 h and used 30 cucumber beetles collected from Davis and starved for at least 12 h. After removing the beetles, I scanned or photographed all leaves with a scale bar and calculated the leaf area removed using ImageJ (NIH) and reconstructions of the initial leaf shape (for *C. quinoa*) and leaf punch diameter for the rest.

Salt as a deterrent

To determine whether NaCl in realistic concentrations would deter cucumber beetle feeding, I presented leaf discs of bladder-removed *C. album* (greenhouse-raised from local seeds), misted with a 473-mL spray bottle (Delta Industries, Hollywood, CA, USA) once with 4 concentrations of NaCl (0, 1, 3, and 5 M; *A. halimus* Na⁺ concentrations in bladders: 1.3–5.4 M; Mozafar and Goodin 1970). While I did not standardize the amount of mist applied, it was consistent across treatments and within an ecologically-relevant range, as bladder mass can be >25 % of leaf mass in small chenopod leaves (unpublished data). Leaves were allowed to air dry for 10 min and presented to 60 locally-collected, starved cucumber beetles (15 per treatment). One day later, I collected the leaf discs, photographed them and measured the area consumed using ImageJ (NIH).

Field tests

On 30 May 2013, I marked pairs of undamaged adjacent leaves on 54 *Atriplex rosea* plants at the UC Davis McLaughlin Reserve, Lake County, CA, USA (8.87476380512704°N 122.44404315948486°W). On each plant, I used 1–4 pairs of leaves, using one undamaged leaf pair per stem and alternating lower/upper leaves manipulated with two treatments: (1) control and (2) bladders and residue removed with a damp soft sponge or a paintbrush. I visually assessed damage categorically (0, <10 %, 10–20 %, etc.) on 12 July 2013.

Analyses

All analyses were performed using R (v.2.13.0, 2011, R Foundation for Statistical Computing). I analyzed the choice test data with two-tailed binomial tests, assuming that consumption of either of the leaves in each pair was equally likely. Individual herbivores that did not feed at all were not included in analyses. To determine whether there was an overall effect of bladders across the tests, I used the method of combining independent tests of significance outlined in Sokal and Rohlf (1969).

I compared leaf area removed in no choice tests using two-tailed *t* tests. In the salt test, I planned to use a one-way ANOVA to compare leaf area consumed, but given the lack

of any consumption in the last two groups, I analyzed this with a two-tailed t test on leaf area removed and compared the numbers of beetles consuming any leaf area with a G test of independence.

Results

Choice tests

Herbivores preferred leaves with salt bladders or without post-bladder rupture residue over intact leaves by a large margin in most tests (Table 1). In no test did more herbivores choose intact leaves. In 96 % (23/24) of tests more herbivores chose cleaned leaves; in 71 % (17/24) of tests, the result was significant at the $p = 0.05$ level. With the method for combining probability from independent tests of significance outlined in Sokal and Rohlf (1969), I found herbivore preference was significantly greater for bladderless leaves ($\chi^2 = 203.1$, $df = 48$, $p = 2 \times 10^{-21}$) across the tests in Table 1. Herbivores similarly significantly preferred non-host plants without added bladders and leaves with DI water over salt or bladder solution (Table 2).

No choice test

Cucumber beetles consumed significantly more leaf matter of bladder- or residue-removed leaves in two of four species tested (Fig. 3). Beetles given quinoa leaves with bladders removed consumed an average of ~50 % more leaf area compared to beetles given leaves with intact bladders, but the result was not significant (two-tailed t test, $n = 30$, $p = 0.066$). Beetles consumed an average of ~80 % more leaf area from bladder-removed leaves compared to leaves with intact bladders for *A. rosea* (two-tailed t test, $n = 30$, $p = 0.046$). Beetles ate slightly more bladder-removed *A. prostrata* leaf tissue than intact leaf tissue in both trials (Davis and McLaughlin plants), but the effect sizes were small and the results were nonsignificant (two-tailed t test, $n = 30$, $p = 0.68$, 0.51 , respectively). Beetles consumed greater than nine times more leaf area of *A. lentiformis* without bladder residue than that of intact leaves (two-tailed t test, $n = 30$, $p = 0.001$).

Salt as a deterrent

Significantly more cucumber beetles fed on leaves with DI water added compared to 1 M NaCl (Fig. 4: G test, $df = 1$, $p = 0.001$). The beetles which fed in the DI group ($n = 14$) consumed significantly more leaf area than those which fed in the 1 M group ($n = 6$) (two-tailed t test, $p = 0.01$). No beetles fed upon leaves treated with 3 or 5 M solutions.

Field test

Of the 54 plants, 22 experienced more damage on the leaves within pairs without bladders, 5 on control (with bladders), 6 had equal, and 21 had no damage. Excluding the plants without damage or where visual assessment was not conclusive, plants suffered greater damage on manipulated leaves within a plant (two-tailed binomial test assuming equal likelihood, $n = 27$, $p < 0.001$).

Discussion

In the majority of choice tests, herbivores preferred leaves without salt bladders, without residue, or with water significantly more than control leaves (Tables 1, 2). In no choice trials (Fig. 3: N2, 4), herbivores removed significantly more leaf area on leaves without bladders than those with bladders in two of four species. Dissolved salt (NaCl) added to leaves was a significant feeding deterrent (#29; Fig. 4). In the field, bladder-removed leaves were significantly more likely to suffer herbivory than adjacent intact leaves. These combined results show that the salt bladders and residue act as feeding deterrents to generalist herbivores. These bladders could deter herbivores physically, chemically, or in both ways.

The presence of bladders or residue on the surface of the leaf could deter herbivores physically by preventing access to leaf tissue beneath the bladder layer or hindering locomotion on the leaf. Both woolly bears and tobacco budworms are medium-large caterpillars, and it is unlikely that salt bladders interfere physically with their feeding, which includes all layers of the leaf with each bite, or with their movement. Cucumber beetles feed primarily on the surface of chenopod leaves, sometimes creating opaque “windows” by feeding on all layers but the lower epidermal layer, thus avoiding the highest density of bladders (personal observation). This avoidance of most bladders could be consistent with either physical or chemical functions.

The tests involving fresh bladders (#6, 7, 10, 11, 13, 15–17, 20, 22, 25, 26) and bladder residue (#1, 3, 8, 18, 22; N2, 4) do not provide a test differentiating chemical and physical mechanisms. The tests involving solutions of bladders (#27) and solutions of residue (#28) excluded the physical mechanism (the solutions were dilute enough to leave no obvious residue when dried); thus the bladders and residue have a chemical deterrent component. The test using a saline solution (#29) and the salt tests suggest that mineral salts are effective deterrents at ecologically-relevant concentrations. While implicating a chemical component, these tests do not exclude a physical defensive mechanism. It is possible that bladders and residue are physical deterrents as well, especially to smaller herbivores (e.g.,

Table 2 Additional choice tests involving bladder, bladder solution, or salt solution additions

No.	Genus	Species	Addition treatment	Herbivore	Addition	Control (DI H ₂ O)	No choice	<i>p</i>
25	<i>Lupinus</i>	<i>arboreus</i>	<i>Blitum</i> bladders	<i>Platyrepia</i>	6	28	2	0.0019
26	<i>Conium</i>	<i>maculatum</i>	<i>Blitum</i> bladders	<i>Platyrepia</i>	7	16	13	0.0466
27	<i>Conium</i>	<i>maculatum</i>	<i>Blitum</i> bladder solution	<i>Platyrepia</i>	4	19	7	0.0013
28	<i>Chenopodium</i>	<i>spinescens</i>	<i>C. spinescens</i> bladder solution	<i>Diabrotica</i>	12	24	0	0.0326
29	<i>Atriplex</i>	<i>prostrata</i>	NaCl (3M) solution	<i>Diabrotica</i>	0	22	0	<0.001

Plant strains are the same as in Table 1. Numbers in the *Addition*, *Control* and *No choice* columns indicate numbers of herbivores which consumed more of the leaf area of the treatment, the control or no or equal quantities, respectively. *p* values are calculated from a two-tailed binomial test assuming equal likelihood of consuming either option

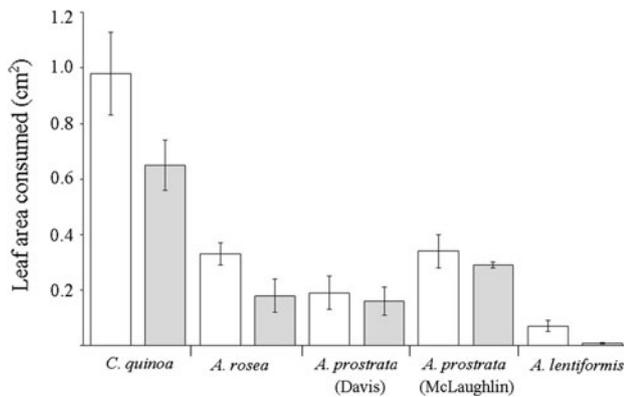


Fig. 3 Mean leaf area consumed (\pm SE) by cucumber beetles in no choice tests. *Dark bars* indicate bladderless leaves (or leaf discs), *light gray* indicate bladder-intact leaves. N1: *Chenopodium quinoa*, N2: *Atriplex rosea*, N3: *A. prostrata*, N4: *A. lentiformis*. Populations are as in Table 1. *p* values, two-tailed *t* tests: N1–N4, *p* = 0.066, 0.046, 0.68, 0.51, 0.001, respectively

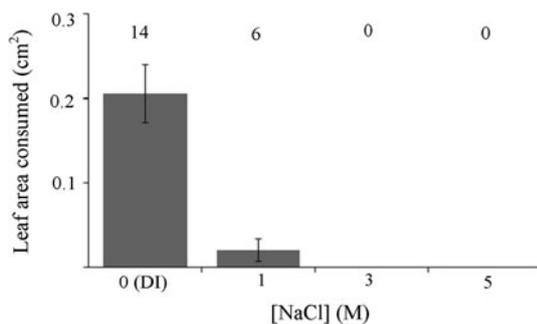


Fig. 4 Mean leaf area (\pm SE) consumed by cucumber beetles in the treatments of the salt deterrent experiment. *Numbers above bars* indicate how many beetles in each treatment consumed any foliage (of 15/treatment). Note that these means include zeros

cucumber beetles). Cucumber beetles chose *C. spinescens* leaves with water over bladder solution at a lower ratio (#28: 2:1) than they chose the same leaves with the bladder residue removed (#22: 6:33:1). This difference in effect

size may be due to additional physical mechanisms operating in the later test.

It is likely that any novel stimulus might be a deterrent when placed on a host plant; the mix of chemical and physical signals that triggers feeding might be upset by this new stimulus. This alternate hypothesis could explain the results on lupine (#25), hemlock (#26, 27), and added salt on *A. prostrata* (#29). This hypothesis is not a viable alternate for the tests on chenopod leaves (#1–24; N1–4; Field), as in these tests the full set of normal leaf signals were given only by leaves with bladders, which were eschewed by most herbivores. Furthermore, budworms and cucumber beetles significantly avoided bladders on certain novel hosts (not from their native range), suggesting that the deterrent is not learned by past association or coevolutionary history with the *specific* plant. This suggests that the substances or physical deterrent involved are general enough to be recognized by a herbivore that has not encountered these plants during ecological or recent evolutionary time.

The no choice tests suggest that intact bladders are highly effective at reducing herbivory. In the field, chenopods generally have bladders and residue intact; the field test suggests these are effective defenses under natural conditions. Within a plant, bladder and residue densities vary (personal observation). The residue is easily washed off with water, through dissolution and dislodgement. Rainfall, condensation, or inundation (for tidal or wetland species) may lessen the effectiveness of these defenses by removing them. For these reasons, these laboratory and field removal tests may be ecologically relevant to naturally arising situations. I thus hypothesize that bladders and residues are more effective defenses in dry climates, where they are likely to remain on the leaf surface. Consistent with this hypothesis (and several alternates, including halophytism), chenopod diversity in North America is negatively correlated with average rainfall, whereas diversity of its sister family, Amaranthaceae, shows no correlation with average rainfall (E.F. LoPresti, unpublished data).

The localization of defenses on the leaf surface may differentially deter herbivores with different feeding methods.

External herbivores probably would incur the highest cost to these external leaf defenses as they would be exposed more readily than leaf miners or stem borers. At one site in the McLaughlin reserve, three chenopod species grow intermixed. The two with bladders, *A. prostrata* and *A. rosea*, suffered moderate herbivory from leaf-mining beetle larvae (*Monoxia angularis* [Coleoptera: Chrysomelidae]) and a much smaller amount by external feeders. In contrast, adjacent *Chenodiastrum* (formerly *Chenopodium*) *murale*, which has very few bladders and fleshy (succulent) leaves was not mined at all by *M. angularis* but was heavily attacked by externally feeding cucumber beetles and armyworms [*Spodoptera exigua* (Lepidoptera: Noctuidae)]. Further observational and experimental work could focus on the potential trade-off between sequestering defenses in leaf tissue and excreting them onto the leaf surface.

It is likely that defensive compounds differ greatly among individuals and populations of chenopods based on a variety of biotic and abiotic factors. In this study, locally-collected *C. album* seeds grown in potting soil left little residue on leaves post-bladder, whereas plants of the same species collected from the shores of a gold mine tailings pond at McLaughlin Reserve (enriched in Mg^{+} , SO_4^{-} , Cl^{-} , B, Ar, Co, Pb, Mn, Sr: Homestake Mining Company and C. Koehler, personal communication) had noticeable post-rupture precipitate on bladdered surfaces. I did not identify the particular defensive compounds in each location and each chenopod species included. Furthermore, it is likely that different herbivores respond to different compounds and many compounds act in concert within a plant. Three classes of compounds are likely candidates in this and other systems involving salt bladders: ionic salts, secondary metabolites, and heavy metals.

The remarkable convergent evolution of salt-removal structures in *Atriplex*-feeding rodents suggests a defensive function of NaCl (Mares et al. 1997). It seems likely that solid ionic salts after bladder burst or the solutions formed in the bladder vacuole could be a part of defense against insects as well; little attention has been paid to any defensive role of these “primary” metabolites, which are necessary for plant function. *Atriplex* spp. concentrate Na^{+} , Cl^{-} , K^{+} , and SO_4^{-} in bladders (Schirmer and Breckle 1982); *C. quinoa* additionally concentrates Mg^{+} , Ca^{2+} , and NO_3^{-} in small quantities (Orsini et al. 2011). Mozafar and Goodin (1970) found that pre-rupture bladders of *A. halimus* in saline growth medium contained >5 M solutions of Na^{+} , which is almost the saturation point and is roughly an order of magnitude more concentrated than seawater. In nonhalophytic growing conditions, the same species concentrated Na^{+} to 1.3 M, still considerably more concentrated than seawater. In the salt deterrent test, even the lowest salt concentration (1 M) lessened herbivory significantly compared to the DI water; high concentrations, but appropriate to *A.*

halimus, completely stopped it (Fig. 4). Furthermore, the *Atriplex lentiformis* in this study was grown on non-saline soil in an arboretum, yet the residue on leaves was quite salty. This residue strongly deterred herbivores (#3 and N4) as did a mid-range *A. halimus* NaCl concentration (3 M) when placed on *A. prostrata* (#29).

In several field studies examining herbivory across natural salinity gradients in salt marshes, certain insect herbivores performed better, or preferred, plants exposed to higher salinities (e.g., Hemminga and van Soelen 1988; Rand 2002), while others performed worse (Hemminga and van Soelen 1992; Martel 1998; Wang and Mopper 2008). Mineral salts may not be the actual compounds responsible for the changes, as physical and chemical plant traits and biotic communities vary in concert along these salinity gradients, but they are likely candidates.

Other ionic salts have been shown to reduce insect herbivory. Leuck et al. (1974) found that spraying dilute mineral salts, including NaCl, KI, and MgO, on bermudagrass, corn, and sorghum deterred herbivory by the fall armyworm, *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae). Their design precluded changes in plant quality as a result of sprayings and they suggest the solutions were dilute enough to not affect plants, pollinators, predators, or parasitoids, only to shift herbivore preference away from the crop plants.

Many heavy metals are defensive compounds in plants (e.g., the elemental defense hypothesis; reviewed by Boyd 2007). Additionally, Leuck et al. (1974) found dilute heavy metal sprays (B, Co, Cr, Mn, and Zi) on plants deterred armyworm feeding. Chenopods are known to be extremely tolerant of soil contaminated with heavy metals (Del Río et al. 2002; Walker et al. 2004; Del Río-Celestino et al. 2006; Moogouei et al. 2011); though they are not known as hyperaccumulators. Schirmer and Breckle (1982) speculate that sequestration and excretion via the bladder system may account for this tolerance; if this is true, the metals would be localized on the leaf surface (like Leuck’s sprays), where they would be apparent and unavoidable to most herbivorous insects. Other plants excrete metals through salt glands (Weis and Weis 2004) or glandular trichomes (MacFarlane and Burchett 2000); therefore, it is possible chenopods do the same. Preliminary data suggest that *Atriplex rosea* excretes large quantities of Fe and smaller amounts of Zn and Cu through its bladder system (unpublished data).

In conclusion, this study demonstrated a novel function of salt bladders and residue in four genera of chenopods (bladder species probably form a monophyletic group; Fig. 5) in an ecologically important relationship, that of a plant–insect interaction. Chenopod species are important economically as food (e.g., quinoa: *Chenopodium quinoa*), livestock forage (e.g., *Atriplex* spp.), and invasive weeds

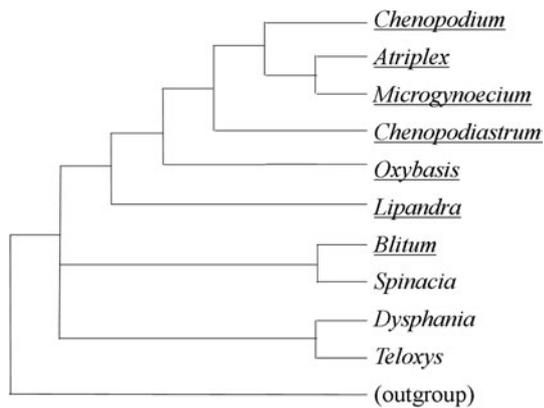


Fig. 5 Current phylogenetic hypothesis of the subfamily Chenopodioideae. Underlined genera possess salt bladders in some or all species (but genera not underlined do not necessarily lack them in all species, I simply found no evidence of them in plants I have examined and the literature). Based on Fuentes-Bazan et al. (2012b)

(e.g., *D. multifida*) (Bassett and Crompton 1978; Fuentes-Bazan et al. 2012a). Understanding the relationships of chenopods with herbivores mediated by these structures could be important for reducing damages to crops via better management practices specific to this family. Bladders and post-rupture residues deterred herbivores in choice tests and led to lower herbivory in no choice tests and the field in both potentially coevolved and entirely novel interactions.

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