

Experimental evidence for an alkali ecotype of *Lolium multiflorum*, an exotic  
invasive annual grass in the Central Valley, CA, USA

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*Abstract*

One of the characteristics of highly invaded ecosystems is that invasive species are often poor invaders of edaphically severe sites, which become refuges for native flora. To investigate the invasive potential of *Lolium multiflorum* (Hick.) into alkali sites in California, an ex-situ reciprocal transfer experiment was carried out using seeds from populations of *L. multiflorum* taken from three sites differing in alkalinity (and inundation), including alkali sink soils (pH 8.5) and sink-edge soils (pH 7.4) located within meters of each other, and non-sink soils (pH 5.0) located several km away. Survivorship, plant height, leaf number and seed production were assessed. In addition, a native composite, *Hemizonia pungens* (Hick.), commonly found on alkali sinks was also sampled at the sink and sink-edge microsites. *Lolium multiflorum* plants grown from alkali sink and sink-edge seeds produced fewer leaves and seeds but taller plants than plants grown from non-alkali seeds, the latter perhaps an adaptation to inundation. Non-alkali genotypes fared poorly in sink soils for all traits, both in comparison to their growth on non-sink soils, and in comparison to the sink and sink edge genotypes. This suggests the existence of *L. multiflorum* ecotypes adapted to inundated alkali sinks, a genotypic difference that occurs on a broad spatial scale (kilometers) but not so obviously on the micro-site scale (meters) between sink and sink-edge populations. These data suggest that edaphic refuges from invasives may be temporary, as invaders with tolerance for these severe sites arrive or evolve.

*Key words:* ecotype, *Lolium multiflorum*, *Hemizonia pungens*, invasive species, reciprocal transfer.

## **Introduction**

Introduced plants have had devastating effects on local biodiversity worldwide (Vitousek et al. 1997, Wilcove et al. 1998). One way these alien species pose a serious threat is when they are competitively superior in to indigenous species. When the local species and alien species have evolved in allopatry, on exposure the indigenous species may lack the ability to successfully compete for light, space, or nutrients. The invasive on the other hand may find itself in a community that lacks similar competitors and therefore has the potential to successfully invade whole habitats.

In particular, the non-forested habitats of the western United States have undergone massive invasions by exotic invasive plant species. The grasslands and woodlands of interior California have had their herbaceous layers nearly completely replaced by invasive annuals (Heady 1988). These invasive exotic annual grasses are a limiting impediment to grassland restoration throughout the western United States (Kay et al. 1981, Dyer and Rice 1997, 1999; Brown and Rice 2000). However, within this sea of exotic species are isolated communities that have resisted invasion. Edaphically extreme plant communities are characterized by a paucity of invaders, and serve as a refuge for native species. These edaphic refuges include serpentine outcrops (Harrison 1999, Gelbard & Harrison 2003, Gram et al. 2004, Brady et al. 2005) and vernal pools (Zedler & Black 2004, Marty 2005).

The alkali sinks of the Central valley grasslands appear to be similar edaphic refuges. In some areas of impeded drainage, low-lying spots are prone to long-term inundation and are strongly alkaline. The plant communities of the background matrix are severely invaded by species such as *Lolium multiflorum* (Italian Ryegrass), which forms nearly monospecific stands. The sinks themselves are largely free of exotic plants and have considerable open ground and

healthy stands of native alkali specialists. However, a few individuals of *L. multiflorum* are found in these sinks, and if they represent a vanguard of alkali ecotypes, may pose a long-term threat to native alkali biodiversity.

Research on native species has examined the role of genetic differentiation in ecotypic adaptation along stress gradients ecotypes (e.g., Kruckeberg 1951, Bradshaw 1984, Bennington and McGraw 1995, Brady et al. 2005), and the particular life history, morphological and physiological traits responsible for fitness differences along stress gradients (Bennington and McGraw 1995, Brady et al. 2005). Although there has been recent research documenting the (slow) invasion of edaphically severe sites by exotic species in both serpentine (Williamson & Harrison 2002) and vernal pool (Gerhardt & Collinge 2003, Black & Zedler 2004) refuges, there has been no research on the potential evolution of exotic ecotypes to the edaphically severe sites that have historically resisted their invasion.

Many introduced species respond genotypically to local environmental conditions and can evolve rapidly to produce an ecotype genetically distinct from their ancestors (Cox 2004). Annuals in particular may have an advantage in rapid specialization due to their short life cycles. Annual invasives with the potential to produce ecotypes rapidly may be a threat in edaphically extreme environments that support rare or endemic natives.

A standard method for investigating ecotypic traits of species is the reciprocal transplant technique (Leiss and Müller-Schärer 2001). If individuals grown in local condition outperform individuals grown in non-local conditions (the "local vs. foreign" criterion of Kawecki and Ebert 2004), this is evidence for evolutionary adaptation to local conditions (Turesson 1922 [from Briggs and Walters 1984], Clausen *et al.* 1940, Cheplick 1988, Primack and Kang 1989,

McGraw and Chapin 1989, Joshi *et al.* 2001, Galloway and Fenster 2000, Montalvo and Ellstrand 2000, Berglund *et al.* 2004).

The aim of this study was to investigate the possibility that *L. multiflorum* at alkali sink sites has produced a locally adapted ecotype and therefore has increased its potential invasive capability at these sites. We analyzed survivorship, growth and fecundity of the exotic invasive *Lolium multiflorum* and native alkali specialist *Hemizonia pungens* ssp. *pungens* taken from alkali sink and sink-edge sites, and in the case of *L. multiflorum* also a non-alkaline site, on different source soils using a greenhouse garden reciprocal transfer experiment.

## **Study species, Study sites, and Methods**

### ***Study species***

*Lolium multiflorum* is a non-native annual grass first introduced to the United States of America by Spanish colonists in the late 1600's (Hannaway *et al.* 1999). Because it is considered a valuable pasture grass, numerous introductions have occurred over the past 300 years, and it has become well established across the continent. It is now considered a pest species in some localities (Hannaway *et al.* 1999), although it is still actively marketed, and is recommended by the USDA for use in rangelands. The California Exotic Pest Plant Council lists it as a potential threat to vernal pools, but lacks sufficient information for a more precise determination (California Exotic Pest Plant Council 1999). The pest status of *L. multiflorum* is compounded by its ability to evolve increased resistance to herbicides (Agriculture and Agri-food Canada 1999). Although *L. multiflorum* prefers well-drained fertile soil, it can survive in poorly drained soil and a period of flooding of up to 20 days if temperatures are low (Hannaway *et al.* 1999). It is tolerant of acidic to mildly alkaline soils (reported range of pH 5.0 to 7.8), and

prefers non-alkaline soils (pH 5.5 to 7.5, (Hannaway *et al.* 1999). *Lolium multiflorum* germinates in the late winter or early spring and thrives in cool wet conditions. Under ex-situ conditions *L. multiflorum* germinates readily without any specific pre-treatments (Hellmers and Ashby 1958, K. Dawson unpublished data).

*Hemizonia pungens* (tar plant) is a composite, native to interior grasslands of the Pacific coast. The subspecies *H. pungens* ssp. *pungens* is an alkali endemic to the Central Valley of California (Hickman 1993). The seeds of *H. pungens* require cold stratification of germination (Dawson, unpublished data). We included it here as a species with a long evolutionary history in these alkali sinks, in contrast to *L. multiflorum*.

### ***Study sites***

Seeds of the two plant species and soils were collected from two sites near Davis, CA. The first is a seasonally inundated pasture located in the central valley of Yolo County, CA approximately 10km northwest of Davis (38° 37' N, 121° 43' W) characterized by alkali pools that retain water well into the spring annual growing season. We sampled both the sink microsites and the non-sink ("sink-edge") microsites. *Lolium multiflorum* and *Hemizonia pungens* occurred in both microsites, although *L. multiflorum* was noticeably sparser in the sinks and *H. pungens* noticeably sparser on sink-edges. *Lolium multiflorum* seeds were also collected from a second site, Bobcat Ranch, a rangeland located on sandy, well-drained non-alkali soils approximately 30 km west of the alkali site (38° 33' N, 122° 03' W). Samples of the three soil types (two alkali microsites and one non-alkali site) were sent to DANR labs at University of California Davis, for chemical and textural analysis.

### ***Reciprocal Transfer***

A common garden experiment was carried out under greenhouse conditions. Sufficient soil was gathered from the same field sites as the seeds of both species. The top 30cm of soil with upper layer of vegetation removed was collected from the alkali site in March 2004. The soil was taken from both alkali sinks and sink-edge microsites, based on pH and visibly different vegetation cover and composition. The pH was initially tested in the field to assist in field differentiation of these two microsites. Soil from the non-alkali Bobcat Ranch site was collected within the same month and again the top 30cm was sampled. The soil was air-dried for 12 days. It was broken into smaller particles and sifted, first to 3mm and then to 1mm. The soil was then heat-treated at 60° C for 72 hr to reduce chances of foreign seeds germinating in the soil during the study. The clay-like nature of the soil from the alkali sites and the small size of the planting pots would have impeded free drainage of the soil. Therefore, to retain the particle size produced by the grinding and sieving process and to emulate “natural” drainage conditions, a polymer solution of 0.02% in de-ionised water was added to the soil in the pots. . The soil was then dried for a further 48 hr at 60°C.

*Lolium multiflorum* was grown in 4x4x7cm plastic tubes, and *H. pungens* was grown in tubes of 3x3x5 cm. A randomized block arrangement was used to accommodate 10 replicates of each of the nine soil/seed combinations (three soil types X three seed types) for *L. multiflorum* and the 4 soil/seed combinations (two soil types X two seed types) for *H. pungens*. The *H. pungens* seed was sown on 10 March 2004 and the *L. multiflorum* on 1 April 2004. Seeds of both species had been collected one year prior and stored at room temperature. The *H. pungens* seeds were cold stratified for several days. All seeds used were soaked in a 10% bleach solution as a fungicide and then rinsed. To assure at least one seedling per pot, three seeds were sown in each *L.*

*multiflorum* pot and five in each *H. pungens* pot. Seedling trays were placed under a mist bench in the greenhouse. Impeded drainage occurred in some (alkali) seedling pots initially but soil drained more effectively after two weeks. All trays were watered to keep the soil moist. Thinning as necessary was carried out on 20 April for the *H. pungens* and on 5 May for *L. multiflorum* to one seedling per planting tube.

The height and number of leaves of each seedling were measured weekly. Height was recorded as the length from the base to the tip of the tallest tiller for the *L. multiflorum* and from the base of the stem to the apical bud for the *H. pungens*. At the end of the growth study (9 June) the *L. multiflorum* and *H. pungens* were moved from the mist bench to a non-misted table in the same greenhouse and only watered 3 times a week, to induce seed set. The number of seeds produced were counted for each individual of *L. multiflorum*. None of the *H. pungens* flowered before the end of the experiment. For analysis we used height and number of leaves produced during vegetative stage and ,for *L. multiflorum*, number of seeds produced during reproductive stage.

### ***Statistical analyses***

We tested the effects of seed source and soil source on plant height, number of leaves, and number of seeds produced (seed set). Seed set was log-transformed for normality. We first tested for local ecotypes by comparing sink and sink-edge seed sources on sink and sink-edge soils for both *H. pungens* and *L. multiflorum*, looking for both main effects and interactions. We then tested for ecotypic differentiation in *L. multiflorum* between the alkali sink site and the non-alkali site. Because there were significant effects of soil source, but not of seed source (see Table 2), we lumped the sink and sink-edge seed sources ("alkali") when comparing to non-alkali



seed sources, but included all three soil sources, again with interaction terms. We examined the relationships between seed set and plant height and leaf number using correlation analysis.

## Results

Compared to the non-alkali soils, the sink and sink-edge soils were characterized by high pH, electro-conductivity, N, P, K, Na, Cl, HCO<sub>3</sub>, Boron, and clay content (Table 1). For most of these traits, the sink-edge soils were intermediate, with values much closer to those in the sink soils than to those in the non-alkali soils. The alkali soil values of pH, Na, Cl, and B are excessive by agricultural standards (Prasad and Power 1997).

Five individuals of *Lolium* (out of 90) and 11 individuals of *Hemizonia* (out of 40) died before reaching maturity. For neither species was there a significant pattern in this mortality with respect to either seed source or soil source.

Height and leaf data provide no evidence of a differentiation of sink and sink-edge ecotypes in *Hemizonia* (Table 2). (*Hemizonia* plants had not yet reproduced by the end of the experiment.) Individuals from both seed sources did grow 50% taller on sink-edge soil than on sink soil (9.1 vs 6.2cm,  $p=0.01$ ).

Analysis of only the sink and sink-edge data reveals an effect of soil source: *Lolium* individuals grown on sink-edge soils produced 60% more leaves than those on sink soils (6.75 vs. 4.20,  $p=0.003$ ). Although not quite statistically significant, there was a suggestion of an ecotype (seed source) effect for seed set, the most fundamental fitness trait (Table 2,  $p=0.08$ , Figure 1).

There was strong evidence of ecotypic differentiation between the alkali and non-alkali populations of *Lolium* (Table 3), in the form of 1) significant seed source effects (for plant

height) and 2) significant seed source by soil source interactions (for number of leaves and seed set). Plants grown from alkali seed sources were nearly twice as tall as those grown from the non-alkali seed source, across all three soil sources (25.7 vs. 13.2cm,  $p < 0.001$ , Figure 2a).

*Lolium multiflorum* plants grown from the alkali sink seed source were significantly more tolerant of sink and sink edge soils than were plants grown from the non-alkali seed source: for both number of leaves and seed set (Figure 2 b,c), there were significant soil source by seed source interaction terms. In both cases, the plants that performed best in either alkali sink or non-alkali soils were plants grown from alkali sink and non-alkali seed sources, respectively.

Across all soil and seed sources, *Lolium* seed set was strongly positively correlated with the number of leaves ( $r^2=0.39$ , d.f. = 85,  $p < 0.001$ ). Seed set was positively correlated with plant height for plants grown from non-alkali seed sources ( $r^2=0.35$ , df = 27,  $p=0.001$ ), but negatively correlated with plant height for plants grown from alkali seed sources ( $r^2=0.19$ , d.f = 58,  $p=0.001$ ).

### **Discussion**

The reported pH tolerance range for *L. multiflorum* introduced into the United States is between 5 and 7.8 (Hannaway *et al.* 1999). The soils of the alkali sink and sink edge habitats are at or beyond the alkaline limits of this range, and are extreme in other soils traits as well (Table 1).

Indeed, all seed sources of both the exotic *L. multiflorum* and native *H. pungens* exhibit lower values of fitness traits (plant size and seed set) on the alkali soils than on the non-alkali soils.

There was a curious pattern for plant height in *L. multiflorum*, which was consistently greater for the alkali genotypes across all soil types. We suspect that this is an adaptation for the extreme inundation that characterized the alkali sites.

Ideally, all fitness measures would be positively correlated, and vegetative measures would be appropriate indirect measures of more direct fitness measures, such as seed set. *Lolium* height was positively correlated with seed set in the non-alkali seed sources, but negatively correlated in the alkali seed sources. One possible explanation of this difference is that in the non-alkali sites, plant height was an indicator of a favorable microsite, and therefore was positively correlated with seed set, but in the alkali sites, ecotypic variation in the resources put into plant height (to escape inundation) comes at the expense of seed set. In any case, this example shows that different fitness measures can become uncoupled in unexpected ways.

Nonetheless, there was clear evidence of ecotypic differentiation between the alkali and non-alkali populations of *L. multiflorum*. In particular, the alkali seed sources performed better on alkali sink soils than did non-alkali seed sources, fulfilling the critical "local vs. foreign" criterion of Kawecki and Ebert (2004). There was even a suggestion of more local alkali ecotypes, because seeds from the sink microsites maintained their fecundity on the severe sink soils, whereas the sink edge seed sources experienced a 37% decline in seed set (from 9.0 to 5.7 seeds/plant; Figure 1). This ecotypic difference was not quite statistically significant, but in precisely the same direction as expected. If confirmed, this would represent ecotypic differentiation on the scale of only a few meters. Although such fine-scale ecotypic variation has been found in similar pools in an insect-pollinated, largely selfing species (Linhart 1988), for a wind-pollinated, outcrossing species like *L. multiflorum*, one might expect limited ability to maintain ecotypes over very short distances.

We do not know if these forms of ecotypic differentiation are due to recent evolution *in situ*, or to the differential arrival and survival of different genotypes (c.f. Vasquez et al. 2005). The fact that *Lolium multiflorum* has been introduced many times, from a variety of different

ecotypes, certainly raises the latter possibility. However, the existence of ecotypes in this study that tolerate pH levels (8.4) far higher than even the extremes reported for *L. multiflorum* in the literature (Hannaway et al. 1999) does suggest local evolutionary change after introduction.

Conservationists and restorationists have long recognized, and taken encouragement from, the fact that edaphically severe sites are often resistant to invasion, and become refuges for native endemic plant species. Recent research, however, has documented the ability of exotic species to invade such sites, albeit slowly (Williamson & Harrison 2002, Gerhardt & Collinge 2003, Black & Zedler 2004). Our study suggests that such invasion may be associated with the arrival or evolution of specialized ecotypes. This is ominous news for those trying to conserve, manage, and restore native species in highly invaded ecosystems, and may provide greater impetus to catch invaders early, not merely as a more cost-effective control measure (Chornesky and Randall, 2003), but to slow or even prevent the evolution of local ecotypes.

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Tables:

Table 1. Soils analysis

<b>Soil trait</b>	<b>Sink</b>	<b>Sink-edge</b>	<b>Non-alkali</b>
<b>pH</b>	8.5	7.4	5.0
<b>EC (dS/m)</b>	2.87	2.11	0.21
<b>Na (meq/L)</b>	26.3	18.6	0.6
<b>Cl (meq/L)</b>	8.5	11.4	1.0
<b>HCO<sub>3</sub> (meq/L)</b>	5.1	4.5	0.9
<b>CO<sub>3</sub> (meq/L)</b>	1.4	<0.1	<0.1
<b>SAR</b>	41	35	1
<b>B (meq/L)</b>	39.1	19.9	0.1
<b>NO<sub>3</sub>-N (ppm)</b>	4.3	3.1	0.2
<b>Olsen-P (ppm)</b>	23.2	38.6	5.2
<b>X-K (ppm)</b>	286	337	104
<b>Sand (%)</b>	16	13	61
<b>Silt (%)</b>	39	35	23
<b>Clay (%)</b>	45	52	16

Table 2. Summary of ANOVA analysis for the effects of seed source and soil source on plant traits of *H. pungens* and *L. multiflorum*, in alkali sink and sink-edge populations.

	Seed source		Soil source		Seed * soil	
	F	p	F	p	F	p
<b>L. multiflorum</b>						
Height	0.43	0.52	2.14	0.15	0.12	0.73
Number of leaves	0.32	0.58	<b>10.39</b>	<b>0.003</b>	0.10	0.75
ln (seed set)	<b>3.20</b>	<b>0.08</b>	0.90	0.35	1.11	0.30
<b>H. pungens</b>						
Height	0.18	0.68	<b>6.99</b>	<b>0.01</b>	0.08	0.78
Number of leaves	0.64	0.43	0.31	0.58	0.31	0.58

Table 3: Results of ANOVA analysis comparing the effects of alkali and non-alkali seed sources, and three soil sources (alkali sink, sink-edge, and non-alkali), on plant traits of *L. multiflorum* .

a) Plant height

<b>Source</b>	<b>df</b>	<b>Sum of squares</b>	<b>F ratio</b>	<b>p</b>
Seed source	1	2777	26.56	<b>0.022</b>
Soil source	2	833	3.98	<b>&lt;0.0001</b>
Seed*Soil	2	15	0.07	0.93
Error	79	8258		

b) Number of leaves

<b>Source</b>	<b>df</b>	<b>Sum of squares</b>	<b>F ratio</b>	<b>p</b>
Seed source	1	9.6	1.07	0.30
Soil source	2	510.7	28.51	<b>&lt;0.0001</b>
Seed*Soil	2	228.0	12.73	<b>&lt;0.0001</b>
Error	80	1327.0		

c) ln (seed set)

<b>Source</b>	<b>df</b>	<b>Sum of squares</b>	<b>F ratio</b>	<b>p</b>
Seed source	1	0.67	2.10	0.15
Soil source	2	6.20	9.80	<b>&lt;0.001</b>
Seed*Soil	2	2.98	4.70	<b>0.01</b>
Error	79	25.00		

Figure captions.

Figure 1. The effects of seed source and soil source on seed set (ln-transformed) for sink and sink-edge *L. multiflorum*. Bars are + one standard error.

Figure 2. The effects of seed source and soil source on plant traits for the alkali (sink and sink-edge) and non-alkali seeds sources of *L. multiflorum* grown in sink, sink edge, and non-alkali soils. Bars are + one standard error.

Figure 1.

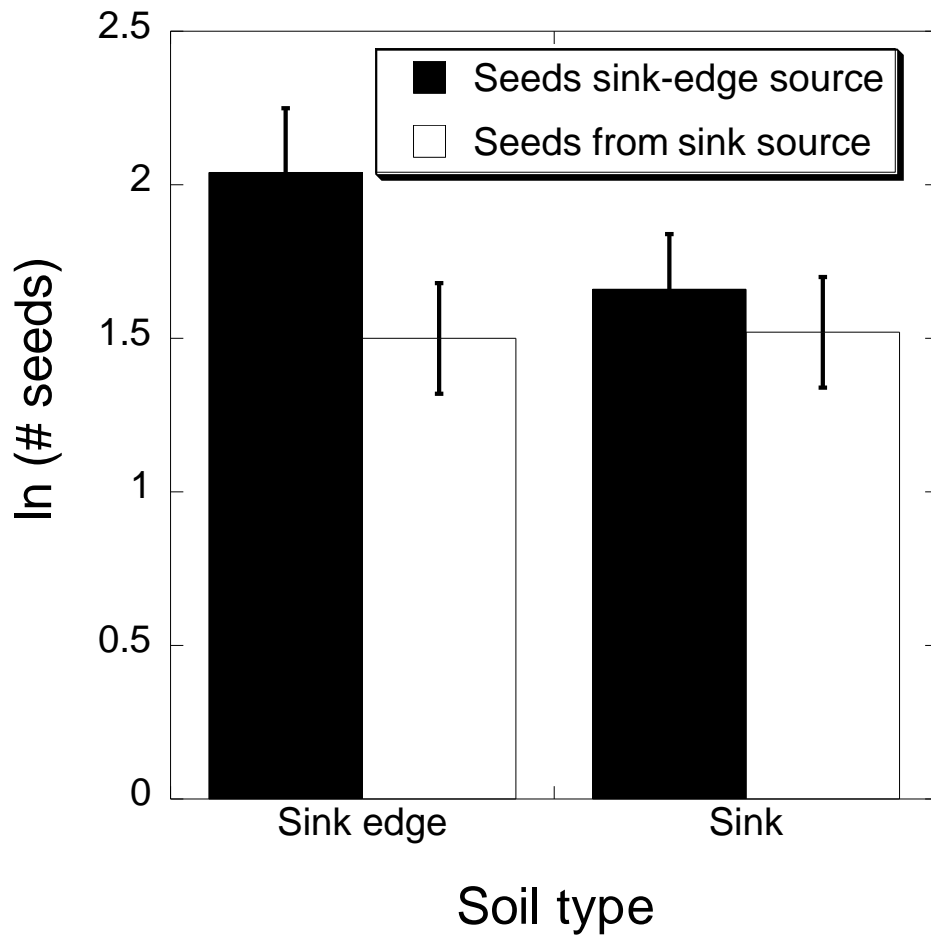


Figure 2.

