

# The importance of nitrogen-fixation for an invader of a coastal California grassland

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**Abstract** Whether a novel trait of an invader directly contributes to increased establishment of that invader is a relatively unstudied question in plant ecology. Nitrogen (N)-fixing shrubs comprise a significant subset of grassland invaders worldwide, which suggests the potential importance of the novel trait of N-fixation in the invasion process. We indirectly tested the importance of N-fixation in the invasion of *Genista monspessulana* (French broom) in a California grassland by alleviating N and phosphorus (P) limitation to the grassland matrix. Grassland productivity was co-limited by N and P; N alone did not release the resident vegetation, and did not affect *Genista* performance. *Genista* was strongly limited by P: seedlings had more nodules, greater leaf N concentration, and higher growth and survival with P additions. When N was added with P, however, growth of the resident vegetation was 50–70% greater than with N or P

alone, accompanied by decreases in *Genista* performance. This suggests that the advantage conferred to *Genista* by N-fixation was dampened when the resident vegetation was released from nutrient limitation.

**Keywords** Novel trait · Nitrogen · Grasses · Legume · Shrub · N-fixation

## Introduction

Many of the dramatic examples of ecosystem impacts of invasive plants involve species that have unique traits compared to residents in the sites they are invading. These novel traits include new life form (e.g. annual grasses in shrubland, D'Antonio and Vitousek 1992; D'Antonio et al. 2000); nitrogen-fixation (N-fixation) (Vitousek and Walker 1989; Witkowski 1991); clonality (D'Antonio et al. 2010) and allelopathy (Callaway and Widenour 2004). The extent to which a novel trait contributes to the initial successful population build up of the invader, however, is not known. Woody N-fixing plants, both native and introduced, provide an excellent test of this question as they have rapidly expanded into some habitats where N-fixers were previously rare or absent over the past century (Lonsdale and Miller 1993; Van Auken and Bush 1997; Vitousek and Walker 1989; Witkowski 1991). While there has been much attention devoted to understanding shrub encroachment of grasslands in general (e.g., climate

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change, grazing and fire exclusion [Archer et al. 1988; Archer 1989]), the specific issue involving the subset of those shrubs that are N-fixers—in particular, the extent to which their success depends on their ability to fix N in assumed N-limited vegetation—has been unexplored.

Because N-fixing plants are, in theory, less reliant on mineral N than are non-fixers, it is reasonable to assume that they should realize their greatest advantage over non-fixers in sites where productivity is constrained by N. Yet, if their own ability to fix N is constrained by their high demand for phosphorus (P), as is known to be the case with many N-fixers (Almeida et al. 2000; Leidi and Rodriguez-Navarro 2000; Wall et al. 2000; Witkowski 1994), then their success may be limited by P availability (but see Redell et al. 1997). In some natural areas, N-fixing legumes do not respond at all to P addition at low N levels that may be characteristic of unfertilized, unmanaged systems (Redell et al. 1997), although others have shown that N-fixation by some species decreases with mineral P levels (Pearson and Vitousek 2002). Most of what we know about nutrient limitation of legumes, however, comes from intensively managed agricultural systems.

In California, several closely related woody legume species are highly successful invaders of grassland ecosystems (Bossard et al. 2000). These include *Genista monspessulana* (hereafter *Genista*), *Cytisus scoparius*, *Spartium junceum*, *Ulex europaeus*, and *Cytisus striatus*, all of which are considered to be among the most invasive of wildland pest plants in the state ([www.cal-ipc.org](http://www.cal-ipc.org)). Because primary production in California grasslands is often N-limited (Grogan and Chapin 2000; Harpole et al. 2007; Hooper and Vitousek 1998) we hypothesized that part of the advantage of these shrubs is their ability to fix N while the surrounding vegetation is N-limited.

We also asked whether *Genista* seedling success and growth was P-limited and sought to evaluate how such limitation would interact with nutrient limitation of the background vegetation. Specifically, we first predicted greater growth of *Genista* in control (i.e., N-limited) plots compared to N addition plots, because N-fixation should provide a greater advantage in the former assuming *Genista* growth was not strongly limited by some other factor. Second, because *Genista* as a legume is likely to be P-limited,

we expected that N-fixation and/or growth of *Genista* would be greater with P addition compared to plots that did not receive P. We simultaneously predicted, however, that if P addition was coupled with N addition and the resident grasses also responded positively to N and P together (because of a tendency toward co-limitation, Harpole et al. 2007), then N + P addition may offset the boost to growth that P would otherwise provide for *Genista* because of greater competition from grasses.

#### Study species and sites

Originally from countries surrounding the Mediterranean Sea and from the Azores, *Genista monspessulana* was introduced to the coast of California in the mid-1800's as an ornamental (McClintock 1985). Its present distribution is at least 40000 ha in California (Bossard et al. 2000).

Our study sites were located within the Golden Gate National Recreation Area in Marin County 10 km north of the city of San Francisco, California (37°52' N 122°31' W). The climate is typical Mediterranean, with the growing season beginning with autumn rains, and continuing until April or May. Temperatures during the annual summer drought are modified by coastal fog. The study area consists of a series of ridge prairies within which are patches of *Baccharis pilularis*, a native shrub, and increasing numbers of *Genista* and *Cytisus scoparius* individuals. Annual grasses, introduced from the Mediterranean region, co-dominate the herbaceous community with native perennial grasses and forbs. The dominant annual grasses are *Bromus diandrus*, *Briza maxima*, *Avena barbata*, and *B. hordeaceus*; the dominant native grasses are *Danthonia californica*, *Nassella pulchra*, and *Festuca rubra*. A small amount (<5%) of bare ground is present, a result of rodent activity. Soils are derived from sandstone and shale and belong to the Barnabe series which consists of shallow, well-drained soils found on uplands in Marin County. These soils are loamy isomesic Lithic Haplustolls, containing approximately 0.36% N (M. Ankovich, unpublished data).

*Genista* began to invade this area approximately 20–30 years ago. Invasion has occurred most abundantly along trails but patches of *Genista* can now be found in open grassland away from the trails. *Genista monspessulana* is the most abundant of the invasive

N-fixers at this site. The density of plants within mature patches at this site is 10.4 adult individuals  $\text{m}^{-2}$  ( $\pm 1.7 \text{ m}^{-2}$ ) but across open grassland individuals occur at less than  $0.1 \text{ m}^{-2}$  (data not shown).

## Methods and materials

In January of 1999, we selected two ridgetop prairie sites  $\sim 1\text{--}2$  km from one another. Both had southwest aspects and slopes of 30–50%, and were selected by the criterion of uninvaded grassland in the vicinity of *Genista* individuals. The sites were dominated by a mix of exotic annual (*Briza major*, *Bromus diandrus*, *Bromus hordeaceus*, *Vulpia myuros*, *Cynosurus echinatus*) and native perennial (*Nassella pulchra*, *Festuca rubra*, *Elymus glaucus*, *Leymus triticoides*, *Festuca californica* and *Danthonia californica*) grasses and had *Genista* adjacent to but not within the grassland. The perennial forbs *Plantago lanceolata*, *Wyethia augustifolia*, *Chlorogalum pomeridianum*, herbaceous legumes *Lupinus bicolor*, and *Vicia* sp., and the sedge *Luzula comosa* comprised the rest of the community.

We established a grid of  $6 \times 4$   $1\text{-m}^{-2}$  plots for a total of 24 plots (6 reps of each treatment) at each of the two sites. Each plot was separated from its neighbor by a 2-m buffer. We offset the beginning of each row by at least one meter from the beginning of its uphill neighboring row to minimize the possibility of nutrient additions affecting plots downslope. Plots were randomly assigned to one of four treatments: +N, +P, +NP, and control (no nutrients added).

In January 1999, we added nutrients at the following rates:  $10 \text{ g m}^{-2} \text{ year}^{-1}$  N as ammonium nitrate ( $\text{NH}_4\text{NO}_3$ ) and  $10 \text{ g m}^{-2} \text{ year}^{-1}$  P as triple super phosphate [ $\text{Ca}(\text{H}_2\text{PO}_4)_2$ ]. We added P at the same rate as N because of the possibility of clay adsorption (Eviner et al. 2000). In subsequent years we added  $5 \text{ g m}^{-2} \text{ year}^{-1}$  for both N and P once at the beginning of each of the 1999–2000 and 2000–2001 growing seasons.

We focused on the seedling stage because the initial stage of invasion (i.e. before the invader starts to affect the environment itself) is a critical stage for the prevention of an invasion (Noble 1989). In October 1999, broom seedlings were transplanted from a nearby area into the experimental plots. We removed blocks of soil containing hundreds of newly

germinated seedlings and randomly selected individuals of similar size (2–3 cm) to be transplanted into the experiment. Nine seedlings were placed in a  $3 \times 3$  grid at the center of each plot. Each seedling was separated from its neighbor by  $\sim 10$  cm. To plant them, a small indentation was made in the soil with a 5 mm diameter wooden dowel, a *Genista* seedling was carefully placed in the hole, and then the soil was tamped down around it. Seedlings were watered every 2–3 days during the first 2 weeks after transplanting ( $\sim 1$  L per plot), and there was no mortality during this period. Seedlings were censused at four time points during the study (February and May 2000; January and June 2001) for mortality and growth.

Resident grassland species were harvested for total aboveground biomass in June of 1999, 2000, and 2001. In each  $1\text{-m}^2$  plot, three  $225 \text{ cm}^2$  subplots were randomly selected in the 25 cm-wide strip surrounding each  $0.25 \text{ m}^2$  inner plot where *Genista* seedlings grew. All plant material was cut at the soil level and then separated into the following functional groups: annual grass, perennial grass, legume (non-broom), annual forb, and perennial forb. Material was not returned to each subplot, but locations noted and marked with small flags so that subsequent harvests would not occur in those same locations. To avoid overestimation of any single year's production by including senescent material (i.e., litter) from the previous year, we attempted to separate this material from the current year's production. This litter material was not analyzed further.

During the final (June 2001) harvest soil cores ( $\sim 5$  cm diam  $\times$  10 cm depth) were collected to estimate total root biomass of the grassland species. One core was taken from each of the three subplots where aboveground biomass had been harvested. Cores were taken back to the laboratory and washed with deionized water in a hydropneumatic elutriator that uses airflow to separate soil material from roots within water (Bel-Art Products, Pequannock, New Jersey, USA). No distinction was made between live and dead roots. All above- and belowground plant material was dried at  $65^\circ\text{C}$  for 48 h. All grassland species responses (biomass, etc.) are expressed on a  $\text{g m}^{-2}$  basis.

In June 2001, *Genista* seedlings were harvested and all nodules were removed from a subset of seedlings (50–100% from each plot). Above- and belowground parts of each seedling were separated.

Soil was washed from roots with deionized water, and plant parts were dried at 65°C for 48 h. After drying, leaves were separated from stems and weighed separately. Leaf material was ground in a Wig-L-Bug amalgamator (Crescent Dental, Lyons, Illinois, USA). Five to six milligram of ground and dried material was then weighed into tin capsules and analyzed for total N by combustion on a Carlo Erba NA 1500 CHN analyzer (Fisons Instruments, Beverly, Massachusetts, USA). Following drying, total nodule biomass for each seedling was weighed and recorded. This value was kept separate from seedling root biomass. Root lengths were also recorded for each seedling. Additionally, plot-level mortality for seedlings was analyzed by calculating the percentage of seedlings that died between the 2000 harvest and 2001 harvest. For all *Genista* response parameters (with the exception of mortality), averages were calculated for each plot using surviving seedlings.

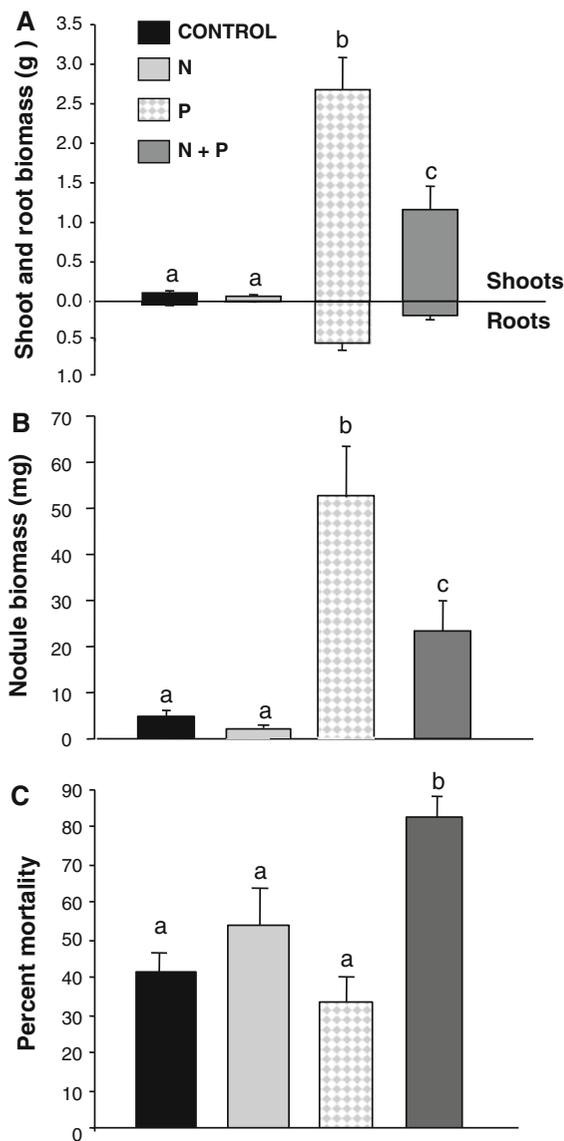
### Statistical analyses

A general linear model with repeated measures for each plot across time was used to analyze total grassland production over the 3 years of fertilization, with N, P, and time as fixed main factors, and block (or site) as a random factor. A similar general linear model analysis was used on all *Genista* growth parameters, but without the main effect of time as these parameters were measured only at the end of the study. Planned contrasts were used to compare each nutrient addition treatment to the control for grassland vegetation, but all pairwise comparisons were made for *Genista* response to fertilization. Linear regression was used to examine the relationships between grassland aboveground biomass and both *Genista* aboveground biomass and percent mortality at the end of the experiment. *Genista* biomass was log-transformed before analysis then back-transformed for presentation. All statistical analyses were conducted using SAS 9.1.3 (SAS Institute 2004).

## Results

### *Genista* response to fertilization

*Genista* seedling biomass (both root and shoot) and nodule biomass were significantly greater with P



**Fig. 1** Mean *Genista* per seedling response to fertilization at the end of 2 years' growth: **a** shoot and root biomass; **b** nodule biomass; and **c** percent mortality. Error bars represent 1 SE. Bars with different letters are significantly different, determined by pairwise comparisons ( $P < 0.05$ ). Letters are the same for roots as for shoots in panel A. The average root biomass in the +N treatment is small but non-zero. ANOVA results are shown in Table 1

addition compared to all other treatments (Fig. 1). Seedlings in N + P plots also had significantly greater growth compared to those in control plots, but at approximately half the rate as with P alone (Fig. 1). There was no main effect of N alone on seedling growth parameters (Fig. 1, Table 1).

**Table 1** Results from mixed-model ANOVA on *Genista* growth variables

	N		P		N*P	
	F	P	F	P	F	P
<i>Genista</i> aboveground biomass	10.68 <sub>1,39</sub>	<b>0.002</b>	59.22 <sub>1,39</sub>	<b>&lt;0.001</b>	9.57 <sub>1,39</sub>	<b>0.004</b>
<i>Genista</i> root biomass	13.15 <sub>1,39</sub>	<b>0.001</b>	41.77 <sub>1,39</sub>	<b>&lt;0.001</b>	11.03 <sub>1,39</sub>	<b>0.002</b>
<i>Genista</i> nodule biomass	8.12 <sub>1,35</sub>	<b>0.007</b>	38.27 <sub>1,35</sub>	<b>&lt;0.001</b>	5.51 <sub>1,35</sub>	<b>0.025</b>
<i>Genista</i> mortality	18.16 <sub>1,46</sub>	<b>&lt;0.001</b>	2.07 <sub>1,46</sub>	0.1569	6.45 <sub>1,46</sub>	<b>0.015</b>
<i>Genista</i> foliar %N	1.47 <sub>1,39</sub>	0.233	9.44 <sub>1,39</sub>	<b>0.004</b>	2.27 <sub>1,39</sub>	0.140

Bold font indicates statistically significant treatment effects ( $P < 0.05$ )

Nodule response mirrored overall biomass response of *Genista* (Fig. 1b). Because the number of nodules had the same pattern as nodule biomass, we report only the latter here. Nodule biomass was greatest in P alone plots, and significantly greater than that in N + P plots with approximately 40% more biomass (Table 1). Seedlings in N + P plots had greater nodule biomass and numbers compared to control, however (planned contrasts,  $P < 0.05$ ). Seedlings in N and control plots, by comparison, were relatively unnodulated (Fig. 1b).

Mortality rates of *Genista* seedlings were significantly affected by fertilization of the grassland, with the response to N depending on P (Table 1). Planned contrasts showed that average mortality rates did not differ across control, N alone and P alone: these treatments had ~35–55% mortality by the end of the experiment. In N + P plots, however, mortality rates were over 80% (Fig. 1c).

### Grassland response to fertilization

The resident grassland species response to N depended on P (significant N\*P interaction term, Table 2). Grassland plots receiving both N and P had the greatest biomass increase relative to control plots (Fig. 2); planned contrasts showed that only this treatment was significantly greater than control. This effect was consistent across time (i.e., no significant interaction of time with N or P, Table 2). Together, annual and perennial grasses comprised the largest proportion of total ANPP (from ~50 to 90%).

Total belowground grass biomass at the end of the final growing season was not significantly affected by any nutrient addition ( $P > 0.05$ , Table 2), with values ranging from ~150 g m<sup>-2</sup> in control plots, to ~200 g m<sup>-2</sup> in N + P plots (Table 3).

**Table 2** Results from mixed-model ANOVA on total grassland biomass over 3 years

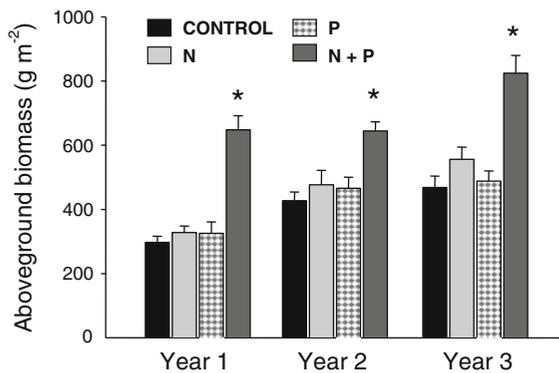
	DF	F	P
Aboveground grassland NPP			
N	1	67.06	<b>&lt;0.001</b>
P	1	47.12	<b>&lt;0.001</b>
N*P	1	29.56	<b>&lt;0.001</b>
Time	2	27.21	<b>&lt;0.001</b>
Time*N	2	1.94	0.147
Time*P	2	1.01	0.368
Time*N*P	2	1.41	0.247
Error	137		
Belowground grassland NPP			
N	1	1.47	0.232
P	1	1.59	0.214
N*P	1	0.18	0.674
Error	46		

Belowground NPP was measured only at the end of experiment. Bold font indicates statistically significant treatment effects ( $P < 0.05$ )

### Relationships between *Genista* growth, mortality and grassland productivity

To further examine the effect of release from nutrient limitation for the resident grassland on *Genista* growth, we analyzed *Genista* seedling biomass as a function of total aboveground grass biomass (annuals plus perennials). For plots both with and without P, there was a significant decline in *Genista* size with increasing aboveground biomass of grasses ( $r^2 = 0.64$ ,  $P = 0.003$  with P addition and  $r^2 = 0.77$ ,  $P = 0.001$  without P addition). Where plots received P, however, *Genista* individuals were larger overall.

Percent mortality of *Genista* seedlings was also related to grass growth across all treatments (data not



**Fig. 2** Mean aboveground response of all grassland species (not including *Genista*) to fertilization across 3 years. Error bars represent 1 SE. Asterisks indicate treatments that are significantly different from the control, as determined by planned contrasts ( $P < 0.05$ ). ANOVA results are shown in Table 2

shown) although only a small amount of the variation was explained ( $r^2 = 0.28$ ,  $P < 0.001$ ). When only P and N + P plots were examined, a greater amount of variation in *Genista* mortality was explained ( $r^2 = 0.46$ ;  $P = 0.002$ ) (data not shown).

## Discussion

We tested the hypothesis that the N-fixing invasive shrub *Genista* would have greater growth in sites where its competitors were N-limited. We found that although N-fixation is a relatively novel species trait in this grassland, *Genista* was strongly P-limited and its competitors were co-limited by N and P. Consequently, our original prediction that the advantage of N-fixation should be apparent in N-limited control plots was not supported: there was no treatment effect of added N for *Genista* or the resident vegetation. Indeed, the almost complete lack of nodules in

control and +N plots suggests that in the unfertilized grassland, *Genista*'s initial establishment was little affected by its N-fixation capability and was controlled by other factors. By contrast, where N-fixation potential was high (in +P plots), *Genista*'s growth and survivorship was increased as long as the residents continued to be nutrient limited (P alone plots). This advantage was lost when the background vegetation was released from nutrient limitation (N + P plots). Nodulation patterns paralleled those described above, and were likely driving the growth of *Genista* rather than vice versa (Parker 2008).

The substantial *Genista* growth and nodulation response to P fertilization is consistent with the abundant evidence of P-limitation to legume growth in agricultural and rangeland systems. There is often, however, great variability across legume species (Tang et al. 1998) and genotypes (Loetscher and Hay 1997) in response to P application, with growth responses ranging from dramatic to negligible. Indeed, there are some forage legumes (e.g., *Lotus glaber*) that thrive in P-deficient soils (Torales et al. 2000). For wildland ecosystems there is scant evidence linking P limitation to success of a woody invading species. For example, Witkowski (1994) added P to co-occurring *Acacia* species as seedlings in pots. While both species responded strongly to P addition, these responses were not linked to invasion patterns. We find our results somewhat surprising because the widespread invasion of *Genista* in coastal California would not suggest much, if any, nutrient limitation. Yet it is possible that sites with less P-limitation may be invaded faster and experience greater impacts of N-fixation due to higher nodule biomass and greater *Genista* production compared to other sites.

Despite the clear evidence for P-limitation to *Genista* growth, *Genista* responded less favorably to

**Table 3** Total belowground biomass (all grassland species besides *Genista*), *Genista* foliar N concentration and root depth with fertilization treatment

	Treatment			
	Control	N	P	N + P
Total belowground biomass ( $\text{g m}^{-2}$ )	149.9 (16.9)	168.1 (23.6)	169.3 (14.5)	207.0 (31.1)
<i>Genista</i> Foliar N (%)	2.16 (0.07)	2.18 (0.12)	<b>2.54 (0.05)</b>	<b>2.31 (0.04)</b>
<i>Genista</i> Root depth (cm)	7.66 (0.22)	7.58 (0.39)	13.1 (0.96)	10.8 (0.50)

Each value represents the mean ( $N = 12$  except  $N = 8$  for N + P) with one standard error in parentheses. ANOVA results in Tables 1 and 2, but font is bold to indicate significant treatment effects

P when N was added. While this is likely the result of an indirect effect of N + P on *Genista* via competition with now vigorous grasses, there is also the possibility of a direct negative effect of N on *Genista*. The direct suppressive effect of N on legumes has been well established in agricultural systems: N additions can reduce the proportion of legumes in grass-legume mixtures, and can also reduce nodulation of legumes (Abdel Wahab and Abd Alla 1996; Chapman et al. 1996; Frame and Newbould 1986). High nitrate levels apparently decrease nitrogenase activity and thereby inhibit N-fixation (Mrema et al. 1997; Pate and Atkins 1983). Empirical support for direct N suppression of N-fixation in wildland species is sparse. To our knowledge, there is no field-based study that has explicitly tested N suppression of growth and nodulation in legumes that have not been historically exposed to fertilization or intensive management. As a comparison, resident herbaceous legumes in our study plots (*Vicia* sp. and *Lupinus bicolor*) were not suppressed by N addition; there was, in fact, a four-fold increase in their biomass with N addition relative to control plots (data not presented). The likely cause of the difference between the resident legumes and *Genista* may have been their different growth forms: resident legumes are herbaceous and thus may respond more quickly and positively to N addition, compared to the perennial shrub *Genista*. Further research is needed to establish whether *Genista* individuals eventually reach a size where nodule biomass increases enough that N-fixation accelerates further growth, reproduction, and subsequent population growth. However, in the early stages of establishment, we found little evidence that N-fixation contributes to the initial buildup of *Genista* invasion.

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