1   **Oceanic salt spray and herbivore pressure contribute to local adaptation of coastal**

2   **perennial and inland annual ecotypes of the Seep Monkeyflower (*Mimulus guttatus*)**

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20   **Key words:** natural selection, local adaptation, ecotype, *Mimulus guttatus*

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ABSTRACT

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25   Identifying the environmental factors responsible for natural selection across different habitats is

26   crucial for understanding the process of local adaptation. Despite its importance, only a few

27   studies have successfully isolated the environmental factors driving local adaptation in nature. In

28   this study, we evaluated the agents of selection responsible local adaptation of the monkeyflower

29   *Mimulus guttatus* to coastal and inland habitats in California. We implemented a manipulative

30   field reciprocal transplant experiment at coastal and inland sites, where we excluded

31   aboveground stressors in an effort to elucidate their role in the evolution of local adaptation. We

32   found that excluding these stressors, most likely a combination of salt spray and herbivory,

33   completely rescued inland plant fitness when transplanted to coastal habitat. In contrast, the

34   exclosures in inland habitat provided limited fitness benefit for either coastal or inland plants.

35   We have previously established that low soil water availability belowground is the most

36   important agent of selection in inland habitat. Therefore, our study demonstrates that a distinct

37   set of selective agents are responsible for local adaptation at opposite ends of an environmental

38   gradient.

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47   **INTRODUCTION**

48   The living world is made rich with varied biological diversity. Much of that diversity is the result

49   of natural selection acting upon variation in wild populations. Local adaptation to differing

50   habitats plays a key role in the evolution of morphologically, physiologically, and phenologically

51   distinct intraspecific populations (Clausen 1951; Schemske 2000; Coyne and Orr 2004). Local

52   adaptation can be described as divergence due to contrasting environmental conditions across a

53   species range. This often results in a tradeoff, where home populations have higher fitness than

54   foreign populations in each habitat (Van Tienderen 1997; Kawecki and Ebert 2004). Over time,

55   local adaptation can lead to the evolution of prezygotic and postzygotic reproductive isolating

56   barriers among populations (Rundle 2002; Nosil 2007; Sobel *et al.* 2010). One of the most

57   effective barriers inhibiting introgression among plants is that of ecogeographic isolation,

58   described as the allopatric distribution of populations enforced by local adaptation to divergent

59   ecological and edaphic regimes (Schemske 2000; Ramsey *et al*. 2003; Husband and Sabara 2004;

60   Kay 2006; Sobel 2014). Strong divergent selection and low relative gene flow across a species

61   range can result in the evolution of disparate ecotypes, groups of locally adapted populations that

62   exhibit reproductive isolation, but not to the point that they would be considered separate

63   biological species (Lowry 2012). While local adaptation is now viewed as a cornerstone of the

64   evolution of biological diversity, surprisingly few studies have identified the key environmental

65   variables, or selective agents, that drive its evolution.

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67   Almost a century has passed since Göte Turesson first introduced the concept of ecotypes

68   (Turesson 1922). Since then, various landmark studies have introduced and popularized the use

69   of reciprocal transplant common gardens to determine the prevalence of ecotypes in numerous

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70   species (reviewed in Leimu & Fischer 2008; Hereford 2009). Most of these inquiries have

71   primarily focused on testing the hypothesis that the ecotypes are local adapted, while little work

72   has been done to identify the causative selective agents (Wadgymar *et al*. 2017). Typically,

73   studies of local adaptation have made use of field or laboratory findings and an understanding of

74   regional natural history to make predictions about potential selective agents, whether they be

75   herbivore resistance in aspect-specific stands of *Quercus rubra* (Sork *et al*. 1993), winter

76   temperatures in natural populations of *Arabidopsis thaliana* (Ågren and Schemske 2012), or

77   predator avoidance via substrate crypsis in *Chaetodipus intermedius* (Hoekstra *et al*. 2005).

78   However, many of these works have appropriately expressed caution in inferring selective agents

79   without employing direct experimental manipulation in field reciprocal transplants. Wadgymar *et*

80   *al*. (2017) recently surveyed the local adaptation literature for studies that identified agents of

81   local adaptation in nature through manipulative field experiments and identified just four such

82   studies (Williamson *et al*. 1997; Bischoff *et al*. 2006; Liancourt *et al*. 2013; Maes *et al*. 2014).

83   Only with further manipulative field experiments can we begin to elucidate the broader causal

84   associations between environmental selective agents and local adaptation (Cheplick 2015).

85

86   In this study, we conducted a manipulative field experiment to better understand the

87   environmental variables contributing to local adaptation in the Seep Monkeyflower, *Mimulus*

88   *guttatus*. Native to Western North American, *M. guttatus* has proven valuable to the study of

89   ecological and evolutionary genetics (Wu *et al*. 2008; Lowry and Willis 2010; Friedman *et al*.

90   2014; Ferris *et al*. 2016; Gould *et al*. 2017; Troth *et al*. 2018). Local adaptation has previously

91   been demonstrated among disparate ecotypes of *M. guttatus* across California's coast-inland

92   moisture gradient (Hall and Willis 2006; Lowry *et al*. 2008; Lowry & Willis 2010). Inland

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93   populations endemic to ephemeral streams and seeps exhibit an annual life history, prioritizing

94   seed production as a strategy to escape the seasonal summer drought (Vickery 1952; Lowry *et al.*

95   2008). In contrast, coastal populations have adopted a perennial life history, persisting year-

96   round in long-lived headland seeps under cooler maritime conditions (Vickery 1952). Due to

97   delayed reproductive maturity, plants of the coastal ecotype transplanted to inland habitats fail to

98   flower before the onset of the hot summer drought (Lowry *et al.* 2008; 2010). Though avoidance

99   of the seasonal summer drought is likely the most important factor responsible for homesite

100   advantage of inland populations, the agents of selection underlying homesite advantage in

101   coastal perennial populations have not been explicitly tested.

102

103   Recent work by Kooyers *et al.* (2017) revealed a potent tradeoff between growth rate and

104   phenylproponoid glycoside (PPG) production (a vital class of herbivore resistance

105   phytochemicals) in variable populations of *M. guttatus* across an altitudinal gradient. Paired with

106   evidence that coastal perennial populations generally produce higher relative concentrations of

107   PPGs that reduce herbivory (Holeski *et al.* 2013; Rotter et al. 2018), this could implicate the role

108   of differential herbivore pressure as a biotic agent influencing divergent selection. Additionally,

109   coastal populations have also been found to be more tolerant to salt spray (Lowry *et al.* 2008,

110   2009), a ubiquitous abiotic stressor in coastal habitats. Despite higher levels of herbivore

111   resistance and salt tolerance of coastal perennial populations of *M. guttatus,* it is unknown how

112   much impact those variables have on fitness in the nature.

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114   Here, we conducted a manipulative reciprocal transplant experiment to investigate whether the

115   manipulations of a habitat's aboveground selective agents can restore fitness in maladapted

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116   foreign ecotypes. Exclosures can buffer plants from the detrimental effects of herbivory, salt

117   spray, wind, and adverse temperatures while holding edaphic soil characteristics constant. To

118   narrow the list of candidate selective agents affecting fitness in the field, plots at both coastal and

119   inland sites were protected with agrofabric exclosures. We then evaluated the fitness of both

120   ecotypes at the end of the growing season, comparing relative performance of exclosure

121   replicates to their controlled counterparts. This design allowed us to demonstrate that edaphic

122   soil factors are not among those agents selecting against inland *M. guttatus* recruits in coastal

123   habitat. Rather, some combination of aboveground agents, most likely vegetative herbivory and

124   salt exposure, are crucial for local adaptation of the coastal populations to their native habitat.

125

126   **MATERIALS AND METHODS**

127   **Genotype selection and growth conditions**

128   To determine what combination of selective agents contributes to differential performance in

129   divergent *M. guttatus* genotypes along California's coast-inland moisture gradient, we conducted

130   a manipulative reciprocal transplant experiment. To verify that these effects are replicable within

131   ecotypes, we used accessions from two coastal perennial (SWB and MRR) and two inland

132   annual populations (LMC and OCC). All seeds were derived from a single field mother per

133   accession. LMC and SWB were collected in Mendocino County, CA and have been shown to be

134   locally adapted through previous reciprocal transplant experiments (Lowry *et al*. 2008; Lowry &

135   Willis 2010). OCC and MRR were collected from Sonoma County, CA and have not been

136   previously utilized in field reciprocal transplant experiments (Table 1). Seeds from each

137   accession were gathered from the wild in previous years and stored in the Lowry Lab at

138   Michigan State University (MSU). All accessions were grown at least one generation at the MSU

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139   greenhouse facilities to control for potential maternal effects and bulk seed stores. Since seed

140   bulking can result in multiple generations of inbreeding in cataloged accessions, we chose among

141   those inbred fewer than four generations (Table 1). Though some inbreeding depression was

142   unavoidable, this screening allowed us to confidently negate the worst effects from our study.

143

144   **Table 1.** Geographic locations and inbreeding information of populations used in this study.

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Ecotype	Pop ID	Inbred	Location	Latitude (N)	Longitude (W)
coastal	SWB-11-1	1 Gen	Irish Beach, Mendocino Co., CA	39° 02' 09"	123° 41' 25"
	MRR-13-2	1 Gen	Jenner, Sonoma Co., CA	38° 27' 38"	123° 08' 45"
inland	LMC-24	3 Gens	Yorkville, Mendocino Co., CA	38° 51' 50"	123° 05' 02"
	OCC-31	2 Gens	Occidental, Sonoma Co., CA	38° 24' 57"	122° 56' 13"

146

147   Seeds were sown at UC Berkeley's greenhouse facilities on February 1st 2017. Each accession

148   was sown as a lawn upon corresponding potting flats (54.28 cm L x 27.94 cm W x 6.20 cm H)

149   filled with Sun Gro Horticulture's Sunshine Mix #1 (two trays per accession, eight in total),

150   moistened prior to sowing with deionized (DI) water. Several hundred seeds were sown to ensure

151   that enough germinated for the experiment. Each of the resulting eight flats were subsequently

152   misted with DI water and stored in a cold room at 4°C to stratify. Coastal flats were relocated to

153   the greenhouse after 10 days of stratification, while their inland counterparts remained for an

154   additional week (17 days). Considering the rapid pace at which inland annuals mature relative to

155   coastal perennials, staggering their relocation allowed us to align the life stages in all our

156   genotypes – regardless of ecotype – for planting in the field. Seedlings were germinated under

157   constant conditions, misted daily, and exposed to 16 hours of daylight. All flats were transported

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158   from UC Berkeley's greenhouse facilities to the greenhouses at UC Davis' Bodega Marine

159   Laboratory & Reserve on February 28th 2017.

160

161   **Reciprocal transplant design**

162   To test whether site-specific agents of natural selection select against non-native ecotypes, a

163   reciprocal transplant common garden experiment was planted along California's coast-inland

164   moisture gradient. Ideal coastal and inland sites were selected at two ecological reserves in

165   Sonoma County, CA. Both gardens were planted in seeps inhabited by native populations of *M.*

166   *guttatus*. The coastal garden was planted along a perennial seep at the southern end of Horseshoe

167   Cove (Latitude: 38.315716°, Longitude: -123.068625°; 60.75 m from the ocean) on land

168   managed by the UC Davis Bodega Marine Reserve (BMR) in Bodega Bay, CA (Figure 1). Our

169   inland site was located along the margins of an ephemeral hillside seep (Latitude: 38.575545°,

170   Longitude: -122.700851°; 39.84 km from the ocean) at the Pepperwood Preserve near Santa

171   Rosa, CA. We established three split-plots at each field site.

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173   All six plots were established with the following dimensions: 216 cm L x 84 cm W (Figure 2B).

174   Plots were positioned haphazardly no farther than 2 meters apart, leaving sufficient room for data

175   collection and plot upkeep. Each plot was cleared of native vegetation prior to transplantation,

176   simulating an artificial landslide event, which are common in California's headland bluffs

177   (Collins & Sitar 2008). All plots were subdivided into two 108 cm L x 84 cm W subplots (6 per

178   site, 12 total) and randomly assigned a treatment: exclosure or shade control (see details of

179   treatments below).

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Bodega Marine Reserve (Coast Site)

Pepperwood Preserve (Inland Site)

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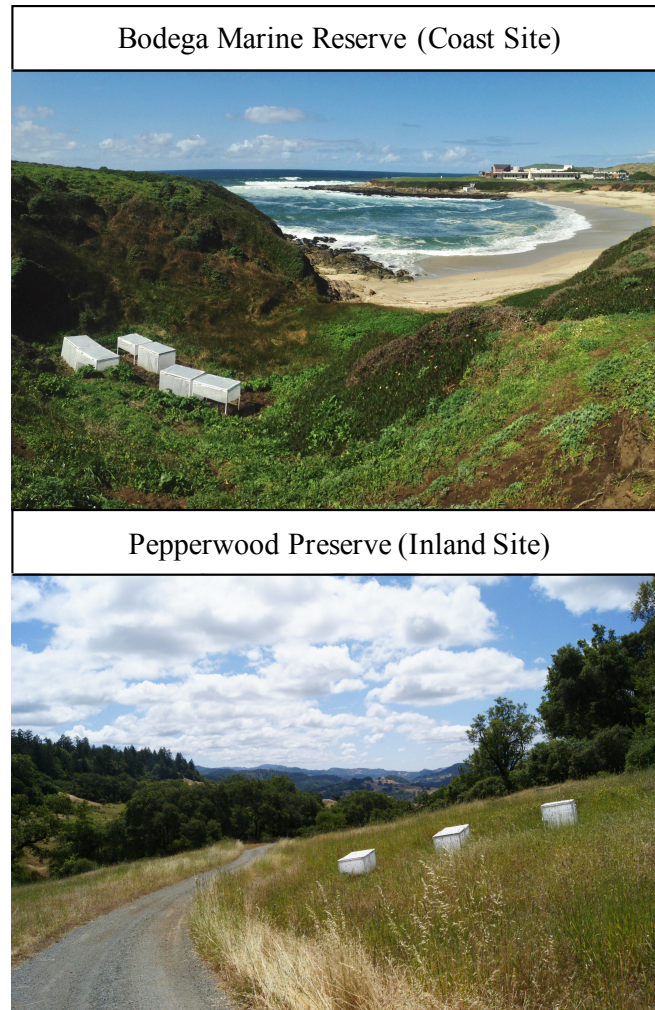
Figure 1. Photos of reciprocal transplant field sites in Sonoma County, CA. The coast garden

was located in a perennial seep running from the bluffs overlooking Horseshoe Cove, down to

the sandy shoreline of the Pacific Ocean. The inland garden was planted along the margins of a

seasonal seep in the Mayacamas Mountains, CA.

186

We transplanted biological replicates at both coastal and inland sites a week after the plants were

relocated to the BMR greenhouses (March 8th 2017). All *M. guttatus* seedlings utilized in this

study were left to mature to the four-leaf stage prior to planting to best ensure transplant success,

but still assess field survival prior to reproduction. 25 replicates per population were planted

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191   haphazardly in a grid within each designated subplot (100 per subplot, 200 per plot, 600 per site,

192   and 1200 total). In all, 26 individuals died within a week of the initial planting (primarily inland

193   replicates at the inland site) and were replaced immediately. All losses within the first week of

194   planting were considered a result of transplant shock, thus justifying swift replacement.

195

196   **Environmental manipulations**

197   To test the combinatorial effects of coastal conditions on population mean fitness, we

198   constructed multiple exclosures with the intent of excluding the aboveground stressors unique to

199   each site – including transient herbivores and oceanic salt spray. Three replicate exclosures (108

200   cm L x 84 cm W x 87 cm H; Figure 2A) were installed at each site (six total) after planting. PVC

201   pipes were used to construct the scaffold of each shelter, consisting of both a ground level and

202   waist height rectangular quadrat attached at each corner by four PVC legs. All joints were

203   reinforced with PVC cement to improve overall rigidity to withstand wind. A third quadrat was

204   mounted with door hinges on the topside of the scaffold to be used as a lid for plot upkeep and

205   data collection. To ensure that biological replicates were sufficiently buffered from the elements,

206   the lid and scaffold of all exclosures were enclosed using medium weight agrofabric – ordered

207   from OBC Northwest, Inc. (Pro-34 1.0 oz./sq. yd. with 70% light transmission). Agrofabric was

208   also applied to the top two thirds of the shade control shelters, leaving the plants exposed to

209   aboveground stressors, while reducing light in a similar way as the exclosures. All holes made in

210   the agrofabric as a result of the zip ties used to fasten them were reinforced with clear repair

211   tape. Lids were sealed to prevent aboveground herbivore intrusion by installing industrial

212   strength VELCRO brand tape strips along the lip of each exclosure. We buried the exclosures in

213   the ground from 8 - 13 cm, depending on location, to limit herbivore entry through the soil.

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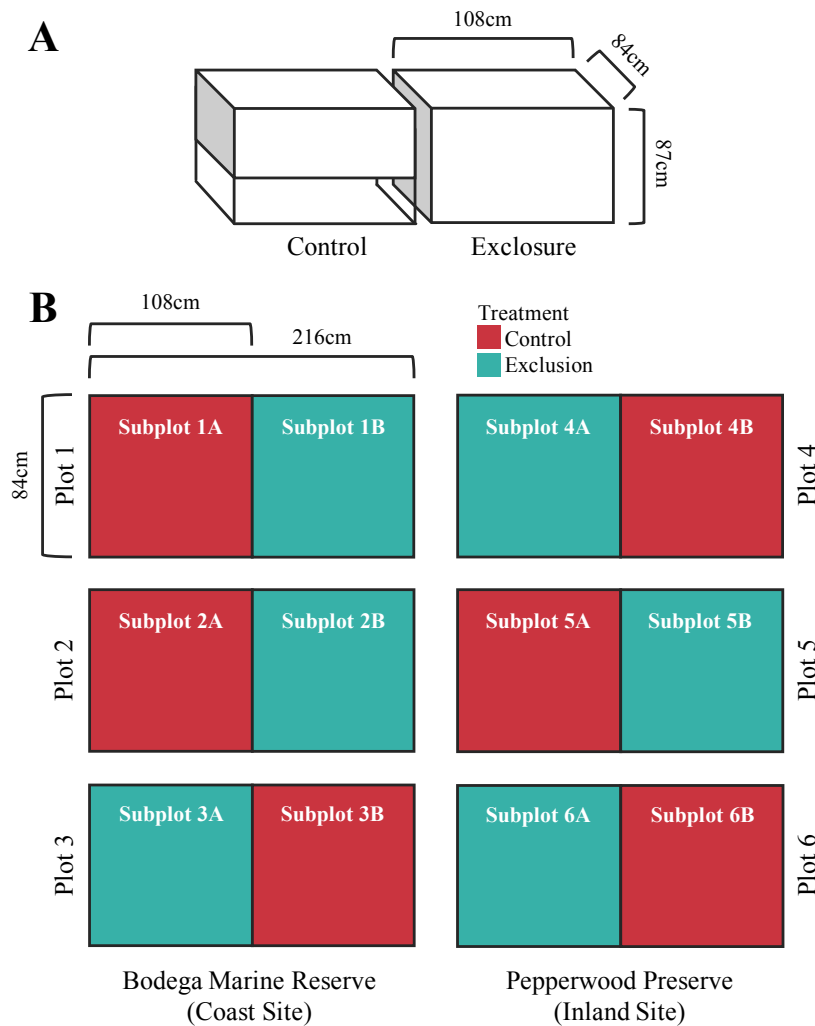
Figure 2. (A) Dimensions of exclosures and shade control shelters used in this study. (B) A

diagram of the split-plot design for both coastal and inland sites.

Effects of field manipulations on fitness

Due to concerns by staff of the field reserves about the potential for introgression of nonnative

genes into the local gene pools, all plants were regularly emasculated. This practice eliminated

the possibility of using flower number or seed set as measures of fitness in this study. Despite

this complication, previous studies on local adaptation in this system demonstrates that selection

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224   is strong enough for survival to be a sufficient fitness measurement in coastal habitats (Lowry et

225   al. 2008; Lowry & Willis 2010). Thus, to study the effects of habitat and exclosure on the

226   performance of both ecotypes, we collected survival data at seven time points throughout the

227   growing season. All aboveground *M. guttatus* vegetation (dead or alive) of each plant was

228   harvested into brown paper bags at the end of the experiment (June 13th – 15th, 2017). These

229   samples were shipped to Michigan State University where they were dried in an oven at 60°C for

230   2 weeks. Each sample was weighed with an analytical balance to quantify dry aboveground

231   biomass, a valuable but imperfect indicator of plant performance and fecundity (Younginger *et*

232   *al*. 2017).

233

234   To test for differential exposure of aerosolized salt spray across treatments at the coast site, salt

235   traps were designed and installed among all plots roughly following Yura (1997) and Yura &

236   Ogura (2006). In total, 6 salt traps were deployed at the coast site (one per subplot) and 2 traps at

237   the inland site (one per treatment). Traps consisted of a four-sided rectangular plastic prism

238   attached to a cylindrical post. Clear vinyl badge holders were clipped upon each face (one per

239   cardinal direction) to act as protective sheaths for Whatman Brand #2 Qualitative Medium filter

240   paper inserts (cut to size: 8.6 cm L x 5.8 cm W). Badge holders have a native cutout to allow the

241   filter paper to absorb all incident salt spray. Traps were left to collect salt for two weeks starting

242   on May 7th, 2017 (24 coastal / 8 inland inserts) and then the filter papers were collected into

243   individual ziplock bags, labelled, and transported back to Michigan State University. Inserts

244   were left to air-dry for 24 hours, placed in Erlenmeyer flasks (1 per trap, 8 total) to soak in 50 ml

245   ultrapure water, and shaken for 1 hour to extract salts. Samples were filtered of all resulting

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246   fibers and debris with Whatman No. 44 filter paper and subsequently analyzed for sodium using

247   inductively coupled plasma optical emission spectrometry (ICP-OES; Olesik 1991).

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249   To quantify the degree to which our exclosures affected herbivore activity, we kept a detailed

250   record of how many replicates had experienced any herbivore damage throughout the extent of

251   our experiment. At the end of the season, all plants were categorized as having experienced

252   herbivory or being unscathed by herbivores. Any replicate that died prior to the accumulation of

253   any obvious herbivore related injuries were not counted as having experienced herbivory. We ran

254   a general linear model (GLM) using a binomial distribution to analyze herbivory in the context

255   of presence / absence. Separate models were run for each site. No random effects were evaluated

256   here, as the goal was simply to determine whether the exclosure treatment had indeed effectively

257   reduced the incidence of herbivory at either site.

258

259   Analysis of transplant data

260   To confirm whether some combination of aboveground stressors contribute to fitness across

261   transplant sites, our data were analyzed using an ASTER modeling approach (Geyer *et al*. 2007;

262   Shaw *et al*. 2008). ASTER is a module developed for the statistical program R that provides a

263   powerful tool for combining multiple fitness components with different probability distributions

264   into a single analysis. The power of ASTER lies in its ability to calculate an expected fitness

265   value for all biological replicates given the order and interdependence of each fitness component.

266   We used ASTER to analyze a composite of 8 fitness components: survival to weeks $1 - 7$, all

267   modeled as Bernoulli (0 or 1), and the final harvested aboveground biomass, here modeled as a

268   normal distribution. Due to coding constraints in ASTER, any replicates with a non-zero mass

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269   that died before the final observation date (measured post mortem) were scored as a zero for

270   biomass. Likelihood ratio tests were constructed by comparing nested null models to test

271   alternative hypotheses.

272

273   We employed a generalized linear mixed modeling approach (GLMM) separately, as an

274   alternative to ASTER because the ASTER module does not readily allow for mixed models with

275   random effects. Thus, the GLMM approach aided us in confirming our ASTER results. Site

276   specific models were developed in lieu of a more comprehensive model, as we were less

277   interested in the effects of site on fitness, but rather the effects of treatment within each site.

278   Response variables included survival to harvest (week 7), modeled using a binomial distribution,

279   and total biomass accrued. Since there is no proper modeling distribution for zero-inflated

280   continuous data in the GLMM framework (data ASTER is quite useful at dealing with), we

281   treated all zero biomass values as missing data and removed them from the analysis. Analysis of

282   the remaining individuals was modeled using a gamma distribution. The following GLMM

283   scaffold was developed for all combinations of site and response (4 models in total): Response ~

284   Treatment + Ecotype + Treatment:Ecotype + (1|Plot) + (1|Accession). While both ASTER and

285   GLMM modeling approaches have limitations, they complement each other's weaknesses and

286   together can increase the confidence in interpretation of results.

287

288   **RESULTS**

289   **General patterns across field sites and treatments**

290   Overall, 89% of transplants survived to harvest at Pepperwood (inland site) and 73% survived at

291   Bodega Bay (coastal site). Exclosures and control subplots at the inland site saw comparable

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292   survival rates, with 90% in the former and 88% in the latter. However, survival diverged

293   markedly at the coastal site with 97% survival in exclosures and 49% in shade control subplots.

294   This vast disparity is driven mostly by discrepancies in ecotype performance dependent on

295   subplot treatment. Plants at the inland site were generally small, having an average biomass of

296   about 0.07g. In contrast, plants at the coastal site had robust growth, ending the season with an

297   average biomass of 1.16g.

298

299   Coastal field site

300   There were striking differences in the responses of coastal and inland accessions to the exclosure

301   treatment at the coastal field site. At the coast, 87% fewer inland control replicates survived until

302   the end of the experiment than the coastal control plants. The few inland individuals that did

303   survive in the control plants were generally small (Fig. 3A,B). In contrast, the inland plants

304   within exclosures experienced nearly the same survival rates and had a similar mean biomass as

305   coastal replicates under either treatment (Fig. 3A,B). Our GLMM approach found a significance

306   of ecotype x treatment interaction at the coast field site ($P < 0.0001$; Table 2), confirming that

307   differential fitness performance between ecotypes was dependent on treatment.

308

309   ASTER modeling generally confirmed the patterns found by GLMM analysis. Not only were

310   there strong treatment effects at the coastal field sire ($P < 0.0001$; Table 3), but treatment was

311   also found to significantly affect expected biomass for each ecotype ($P < 0.0001$; Table 3).

312   Although the exclosure treatment led to an increase in the expected mean vegetative biomass of

313   both ecotypes at the coastal site, the response of inland plants to the treatment was much more

314   dramatic. This is most evident in Figure 4A, where inland exclosure replicates have accrued an

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315   expected mean biomass of 1.58g while inland plants in the control plots had a negative expected

316   biomass (-1.33g) due primarily to low survival.

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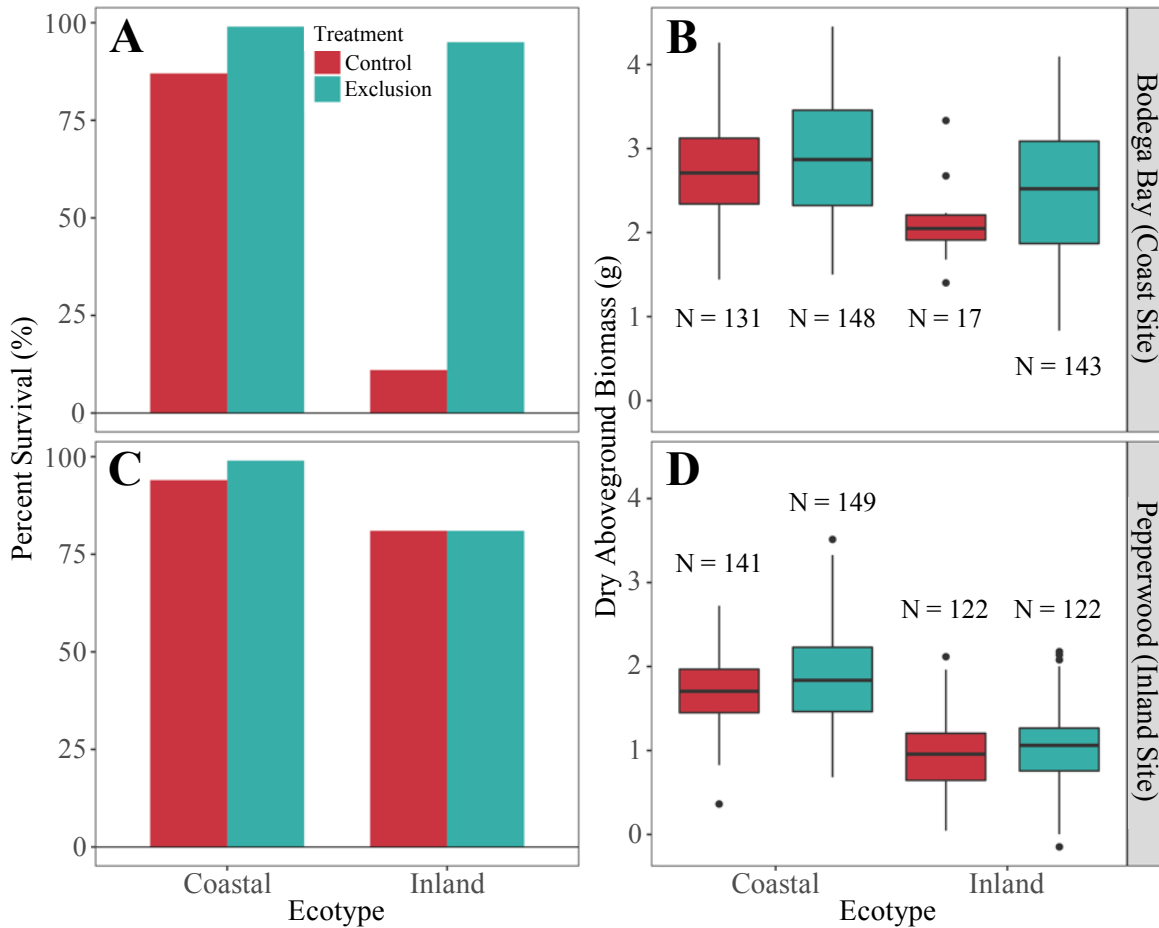
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319   **Figure 3.** Survival and dry aboveground biomass were measured as fitness proxies in all

320   replicates across both reciprocal transplant gardens to model the effect of ecotype, treatment, and

321   ecotype x treatment interactions on in situ performance. Percent survival is displayed in bar plots

322   specific to the coast (A) and inland (C) sites. Box and whisker plots of dry aboveground biomass

323   at the coast (B) and inland (D) sites for plants that survived to harvest (N = sample size of

324   surviving individuals).

325

326   **Table 2.** Results for the following Generalized Linear Mixed Model (GLMM): Response ~

327   Treatment + Ecotype + Treatment:Ecotype + (1|Plot) + (1|Accession). The *P* values for main

328   effects and interactions are provided.

Site	Response Variable	Treatment	Ecotype	Ecotype x Treatment
coast	survival to harvest	0.0005***	<0.0001***	<0.0001***
	dry aboveground biomass	<0.0001***	0.10312	0.0398*
inland	survival to harvest	0.0337*	0.0015**	0.0408*
	dry aboveground biomass	<0.0001***	<0.0001***	0.0308*

329   *P<0.05, **P<0.01, ***P<0.001

330

331   **Inland field site**

332   Ecotype, treatment, and ecotype x treatment interaction all had a significant effect on survival

333   and biomass at the inland site (Table 2). However, the magnitude of these differences was

334   relatively small, with a 19% difference in survival between ecotypes and a 3% difference in

335   survival between the treatments. There were significant effects of ecotype on survival (*P* =

336   0.0015; Table 3) and biomass (*P* < 0.0001; Table 2). Coastal replicates both survived in greater

337   numbers and produced more vegetative biomass than their inland counterparts (Fig. 3C,D).

338   Regardless of these differences, the vast majority plants at this site still survived to harvest.

339

340   There was no significant effect of treatment in our ASTER models (*P* = 0.1376; Table 4).

341   However, the ecotype x treatment interaction was significant for biomass (*P* < 0.0001; Table 4).

342   This interaction is the result of an almost three-fold difference in expected biomass between

343   treatments, with exclosure biomass being greater than that of our control plots (Figure 4B).

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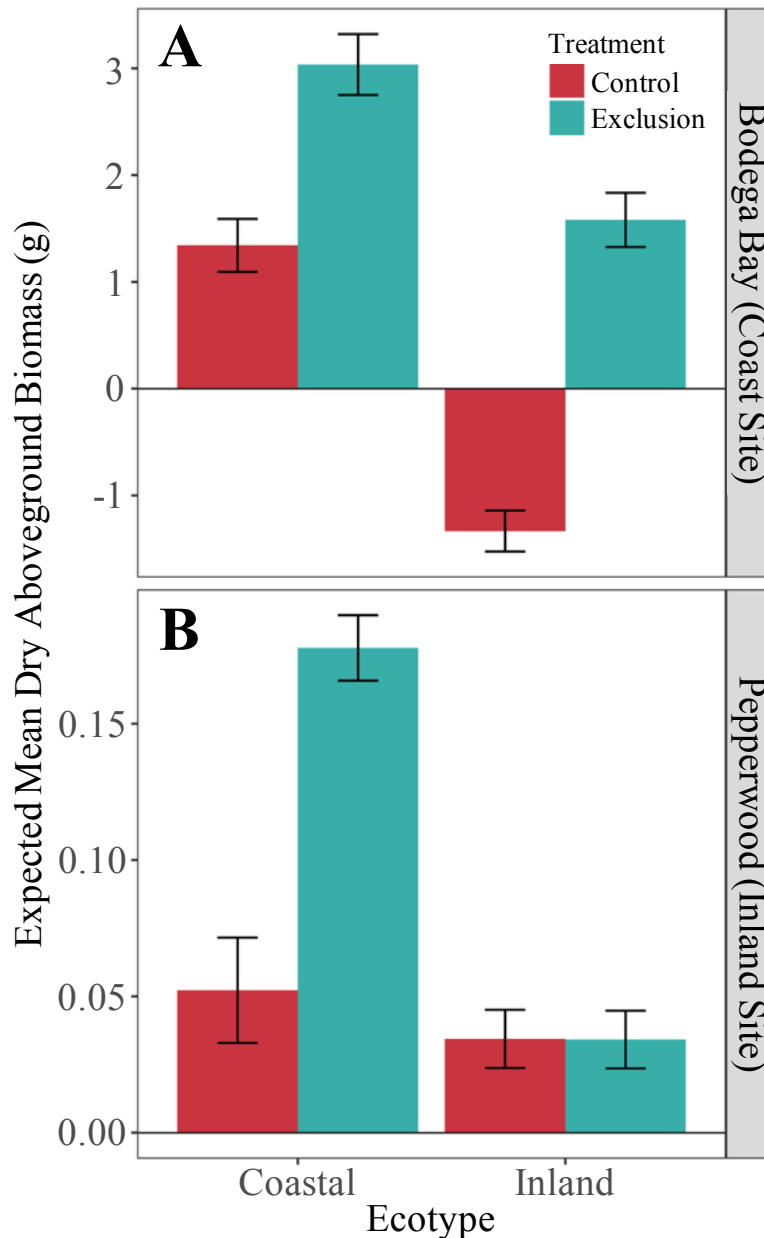
Figure 4. Expected mean dry aboveground biomass of all harvested replicates at the coast (A) and inland (B) sites from the ASTER analysis. Here, expected biomass accounts for the combination of survival with final dry aboveground biomass. A negative expected mean can arise if enough mortality is observed in any particular treatment group. All error bars denote one standard error. Note that scales are different.

351

352 **Table 3.** Analysis of treatment and treatment-by-ecotype interactions using an ASTER-based

353 modeling approach. Our ASTER models analyzed a composite of eight fitness components,

354 including survival from weeks 1 – 7 and a post-harvest measure of dry aboveground biomass.

355 These components were aligned in the following directional graph in order of general causality:

356 survival to week 1 → survival to week 2 → survival to week 3 → survival to week 4 → survival

357 to week 5 → survival to week 6 → survival to week 7 → biomass accrued. All factors were

358 tested by likelihood ratio tests using nested null models.

| Site	Factor Tested	Null df	Alternative df	Null Deviance	Alternative Deviance	Test df	Test Deviance	Test p Value
coast	Treatment	9	10	-1048.00	-930.70	1	117.330	<0.0001***
	Ecotype x Treatment	10	11	-930.70	-903.31	1	27.385	<0.0001***
inland	Treatment	9	10	-541.45	-539.24	1	2.205	0.1376
	Ecotype x Treatment	10	11	-539.24	-503.36	1	35.887	<0.0001***

359 ***$P<0.001$.

360

361 **Quantifying salt spray and herbivory**

362 Quantifying the concentration of accumulated sodium allowed us to establish whether our

363 exclosures had any significant effect on incident salt spray levels in control vs. exclosure plots.

364 Sodium concentrations were found to be elevated in the ambient conditions of control subplots at

365 the coastal site in comparison to any other combination of treatment and site (Figure 5). Salt

366 samples collected from coastal control subplots had nearly a two-fold higher level of sodium

367 over those sheltered within exclosures. There did not appear to be any difference between the

368   control or exclosure treatments at the inland site. Analysis of a 2% nitric acid blank and clean

369   filter control revealed that our solvent and filter slips had small-to-negligible effects on the

370   sodium content of our test samples. This verifies a pattern of ambient salt reduction as a

371   consequence of our exclosure installations.

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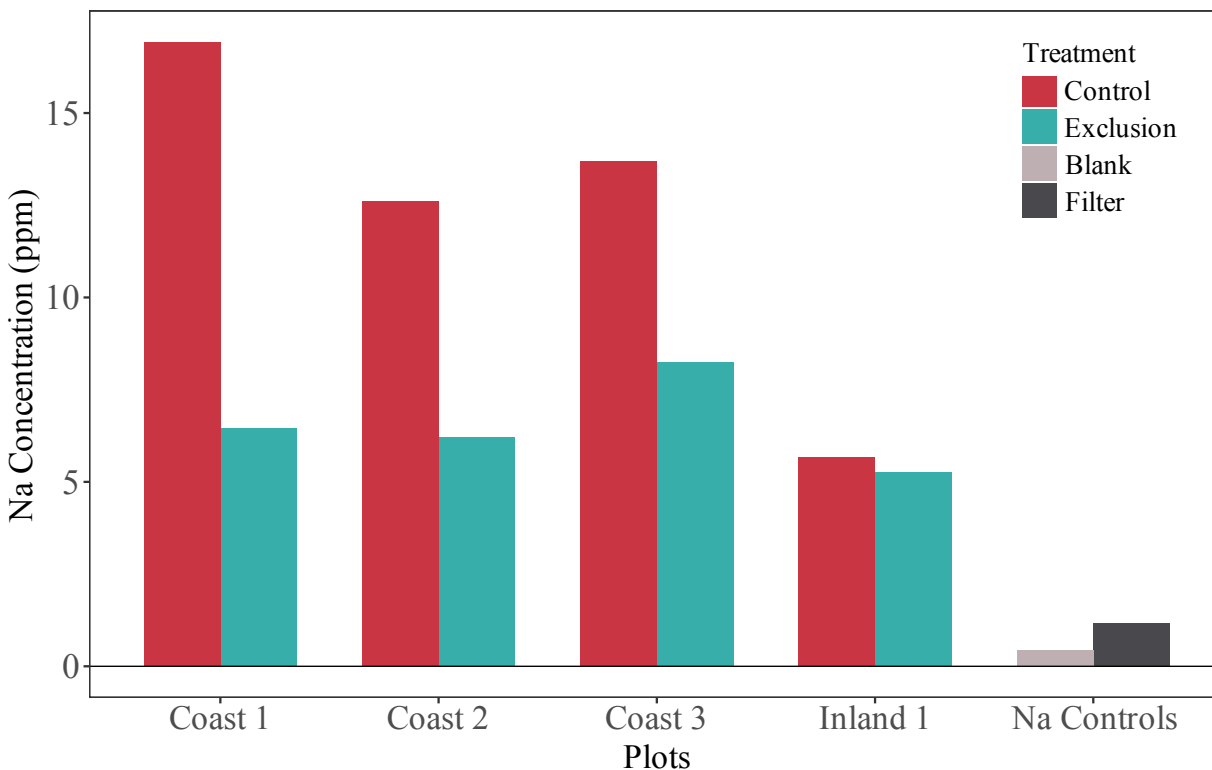
Figure 5. Sodium (Na) concentrations in ppm of all salt trap filter slips left in the field for two weeks, measured using inductively coupled plasma optical emission spectrometry (ICP-OES). Control samples were subject to elements native to each site and plot microhabitat. Exclusion samples were installed within the bounds of their respective exclosure. A blank sample was run consisting only of 2% nitric acid. To gauge whether Na levels were altered in the presence of filter slips, an unadulterated filter control was also analyzed. All coastal plots and one of three total inland plots are shown.

20

381   Similar to our sodium analyses, quantifying the incidence of herbivory confirmed the

382   effectiveness of our exclosures. Treatment had a significant effect on herbivory at the coastal site

383   ($P < 0.0001$), nearly eliminating the incidence of herbivore damage to the plants within the

384   exclosures (Table 4). Neither the ecotype nor ecotype x treatment interaction had a significant

385   effect on herbivory at the coast. Though nearly a quarter of coastal and inland controls

386   experienced some herbivore damage at the coast site, there were few observed instances of

387   herbivory at the inland site regardless of treatment (Table 4). In contrast to the coast site, there

388   was no evident effect of treatment on the incidence of herbivory at the inland site ($P = 0.8105$).

389   Only ecotype had a significant effect on rate of herbivore damage ($P = 0.0189$), although how

390   meaningful this was given the low level of herbivory is doubtful (Table 4).

391

392   **Table 4.** Percentage of plants that experienced herbivory across sites and treatments. %

393   Herbivorized was calculated by dividing the final number of herbivorized plants by the total

394   present in each treatment group.

Site	Ecotype	Treatment	% Herbivorized
coast	coastal	exclosure	0
		control	28
	inland	exclosure	0
		control	29
inland	coastal	exclosure	0
		control	7
	inland	exclosure	1
		control	1

DISCUSSION

395

396   Our results confirm that some combination of aboveground stressors contributes to the adaptive

397   divergence of coastal perennial and inland annual ecotypes of the Seep Monkeyflower, *M.*

398   *guttatus*. While coastal conditions strongly favor the persistence of native genotypes, nonnative

399   inland accessions at the coastal site were shown to be rescued by exclosure treatments. The effect

400   was so strong that fitness was not merely restored, they thrived at levels comparable to their

401   coastal adapted congeners. Our results suggest that coastal populations have likely adapted

402   primarily to withstand the adverse effects of aboveground selective agents including oceanic salt

403   spray and higher levels of herbivory. In contrast, previous studies have found that local

404   adaptation inland annual populations of *M. guttatus* are likely primarily driven by escape,

405   through earlier flowering, from low belowground soil water availability that occurs during the

406   seasonal summer drought (Lowry *et al*. 2008; Hall et al. 2010; Lowry & Willis 2010). Together,

407   these results demonstrate that different selective agents are responsible for local adaptation at

408   opposite ends of an environmental gradient.

409

Patterns of local adaptation

410

411   The most significant result of our study was the striking effect of the exclosure on survival and

412   biomass of inland annual transplants at the coastal field site, despite sharing the edaphic

413   conditions of neighboring control replicates. These exclosures ameliorated all of the detrimental

414   effects of natural selection on non-native transplants at the coastal field site. In contrast, the

415   exclosures did not have nearly as great an effect at the inland field site, where coastal perennial

416   plants survived at similar levels as inland annuals. Our analyses demonstrate that a simple

417   control of aboveground stressors in the field can overcome the environmental variables that limit

22

418   inland fitness at the coastal field site. Thus, some suite of aboveground agents causes the

419   selection that maintains local adaptation of coastal populations of *M. guttatus*.

420

421   The effect of our treatment at the inland field site was unexpected, since hypothesized agents of

422   selection like salt spray are not a factor in this habitat and rates of herbivory are much lower. The

423   elevated biomass of the coastal perennial transplants within exclosure were most exaggerated in

424   two of the three inland plots, coinciding with exclosure subplots planted closer to the interior of

425   the seep where soil moisture levels remains favorable for longer. While this result could be an

426   artifact of experimental setup, the same trend was not evident among inland plants. Therefore, it

427   may be that an unknown aboveground set of selective agent limits the performance of coastal

428   transplants in inland habitats. Future studies conducting detailed quantification of herbivory and

429   other factors will be needed to draw any conclusions about mechanism.

430

431   In contrast to previous findings on this system, survival of both native and non-native genotypes

432   at the inland site remained comparable throughout the entire growing season. While this result

433   appears to be at odds with local adaptation, it was expected. We have shown in previous studies

434   that despite the consistent performance of coastal transplants early in the growing season at

435   inland field sites, nearly all are killed by the low soil water availability of the summer drought

436   before they have the opportunity to flower (Hall et al. 2006; Lowry et al. 2008; Hall et al. 2010;

437   Lowry & Willis 2010). Fast growing inland annuals survive to flower at very high rates at inland

438   field sites. Thus, by ending the experiment before the summer drought, our results did not

439   capture selection imposed by seasonal drought.

440

441 **Salt spray and herbivory as agents of selection at the coastal field site**

442 Salt stress, whether derived from topical incidence or root uptake, can have a range of adverse

443 effects on plants (Boyce 1954; Humphreys 1982; Griffiths 2006). However, coastal populations

444 of *M. guttatus* – often occurring within a few meters of the wavebreak – are known to have a

445 higher tolerance to topical salt application than inland plants (Lowry *et al*. 2008, 2009). The

446 majority of inland replicates in the control plots at the coast site experienced complete vegetative

447 loss consistent with leaf necrosis due to salt spray. Few inland controls persisted long enough to

448 successfully form floral buds. Further, among all bolting survivors, floral stalks and lateral

449 branches turned brown and produced no healthy flowers, likely as a result of salt spray exposure.

450 Similar instances of premature vegetative senescence were noted in inland replicates within

451 coastal exclosures, but this damage was only limited to those tissues in direct contact with the

452 agrofabric. This tissue was presumably experiencing salt stress as a consequence of contact with

453 oceanic salt that had accumulatd on the walls of the exclosure barriers.

454

455 Our field observations revealed significantly higher rates of herbivory among control replicates

456 of all genotypes at the coast field site, demonstrating the exclusionary power of our agrofabric

457 treatments. Yet there appeared to be no apparent bias towards one ecotype over another. At least

458 for the field season in which we conducted the experiment, mammalian predation by resident

459 California Voles (*Microtus californicus*) made up a significant portion of damage done to control

460 replicates at the coast – most often evidenced by the complete removal of floral stalks and

461 branches. Though plants in the coastal control plots experienced more herbivory than the plants

462 in the exlosures, it did not appear widespread enough to be the sole explanation for such striking

463 fitness differentials across treatments. Overall, some combination of herbivory and salt spray

24

464   have clearly contributed to local adaptation of coastal populations of *M. guttatus*. However, we

465   cannot parse the relative importance of these two factors without further manipulative field

466   experiments.

467

468   **ACKNOWLEDGEMENTS**

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500   LITERATURE CITED

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