OCEANIC SALT SPRAY AND HERBIVORE PRESSURE CONTRIBUTE TO LOCAL ADAPTATION OF COASTAL PERENNIAL AND INLAND ANNUAL ECOTYPES OF THE SEEP MONKEYFLOWER (MIMULUS GUTTATUS)

By

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ABSTRACT

OCEANIC SALT SPRAY AND HERBIVORE PRESSURE CONTRIBUTE TO LOCAL ADAPTATION OF COASTAL PERENNIAL AND INLAND ANNUAL ECOTYPES OF THE SEEP MONKEYFLOWER (MIMULUS GUTTATUS)

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In this study, we used the emergent model system *Mimulus guttatus* to explore the agents of selection that drive local adaptation across California's coast-inland moisture gradient. We implemented a field reciprocal transplant experiment within agrofabric exclosures at sites in coastal and inland Sonoma County, California – minimizing the effects of aboveground stressors in an effort to elucidate their role in the evolution of local adaptation in this system. ASTER life-history modeling and generalized linear mixed modeling approaches were used to analyze survival and dry aboveground biomass as fitness proxies. Despite altering no edaphic conditions, we found that among coastal exclosures, inland replicates were significantly more fit than their control counterparts, essentially rescuing inland fitness outside of their native range. Exclosures provided no fitness advantage for either ecotype at the inland site, aside from a moderate but statistically significant increase in biomass among exclosed coastal replicates. While it is unlikely to know all the agents of selection limited by our exclosures, we found that the number of replicates that experienced herbivory were significantly lower across all exclosures at the coast site. An elemental analysis of Sodium (Na) using salt traps installed at each site also demonstrated a reduction in salt exposure within exclosures. It is likely that some combination of aboveground stressors, likely driven primarily by herbivory and/or salt stress, plays a continuing role in the evolution of coastal *M. guttatus* populations, thus providing a new understanding of how local adaptation is maintained in this model system.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The living world is made rich with varied biological diversity. Much of that diversity is the result of natural selection acting upon variation in wild populations. Local adaptation to differing habitats plays a key role in the evolution of morphologically, physiologically, and phenologically distinct intraspecific populations (Clausen 1951; Schemske 2000; Coyne and Orr 2004). Local adaptation can be described as divergence due to contrasting environmental conditions across a species range. This often results in a tradeoff, where home populations have higher fitness than foreign populations in each habitat (Van Tienderen 1997; Kawecki and Ebert 2004). Over time, local adaptation can lead to the evolution of prezygotic and postzygotic reproductive isolating barriers among populations (Rundle 2002; Nosil 2007; Sobel *et al.* 2010). One of the most effective barriers inhibiting introgression among plants is that of ecogeographic isolation, described as the allopatric distribution of populations enforced by local adaptation to significantly different, often distant ecological and edaphic regimes (Schemske 2000; Ramsey *et al*. 2003; Husband and Sabara 2004; Kay 2006; Sobel 2014). Strong divergent selection and low relative gene flow across a species range can result in the evolution of disparate ecotypes, groups of locally adapted populations that exhibit reproductive isolation, but not to the point that they would be considered separate biological species (Lowry 2012). While local adaptation is now viewed as a cornerstone of the evolution of biological diversity, surprisingly few studies have identified the key environmental variables, or selective agents, that drive these processes.

Almost a century has passed since Göte Turesson first introduced the concept of ecotypes (Turesson 1922). Over the past century, various landmark studies introduced and popularized the use of reciprocal transplant common gardens to determine the prevalence of ecotypes in numerous species (Leimu and Fischer 2008; Hereford 2009). Most of these inquiries have primarily focused on demonstrating the patterns of local adaptation, while little work has been done to identify the selective agents in the environment that result in these patterns (Wadgymar *et al*. 2017). Many such studies have made use of field or laboratory findings and an understanding of regional natural history to make predictions as to the identity of these mechanisms, whether it be herbivore resistance in aspect-specific stands of *Quercus rubra* (Sork *et al*. 1993), freezing period in natural populations of *Arabidopsis thaliana* (Ågren and Schemske 2012), or predator avoidance via substrate crypsis in *Chaetodipus intermedius* (Hoekstra *et al*. 2005). However, many of these works have appropriately expressed caution in inferring selective agents without employing direct experimental manipulation in field reciprocal transplants. Wadgymar *et al*. (2017) recently surveyed the local adaptation literature for studies that identified agents of local adaptation in nature through manipulative field experiments and identified only four such studies (Williamson *et al*. 1997; Bischoff *et al*. 2006; Liancourt *et al*. 2013; Maes *et al*. 2014). Only with further in situ common garden manipulations can one begin to elucidate the broader causal associations between external selective agents and their resultant local adaptation (Cheplick 2015).

In this study we conducted an experiment to better understand the environmental variables contributing to local adaptation in the Seep Monkeyflower, *Mimulus guttatus*. Native to Western North American, *M. guttatus* has proven valuable to the study of ecological and evolutionary genetics (Wu *et al*. 2008; Lowry and Willis 2010; Friedman *et al*. 2014; Ferris *et al*. 2016; Gould *et al*. 2017; Troth *et al*. 2018). Local adaptation has previously been demonstrated among disparate ecotypes of *M. guttatus* across California’s coast-inland moisture gradient (Hall and

Willis 2006; Lowry *et al*. 2008; 2010). Inland populations endemic to seasonal lowland streams exhibit an annual life history, prioritizing seed production as a strategy to escape the seasonal drought (Vickery 1952; Lowry *et al*. 2008). In contrast, coastal populations have adopted a perennial life history, persisting year-round in long-lived headland seeps under cooler maritime conditions (Vickery 1952). Due to delayed reproductive maturity, plants of the coastal ecotype transplanted to inland habitats fail to flower before the onset of the hot summer drought (Lowry *et al*. 2008; 2010). Though California's seasonal droughts are a strong candidate selective agent for adaptation to drier inland habitats, the agents underlying homesite advantage in coastal perennial populations are not as clear.

We considered the role of two hypothesized selective agents in the evolution of coast adapted *M. guttatus*: (1) herbivory and (2) oceanic salt spray. Recent work by Kooyers *et al.* (2017) revealed a potent tradeoff between growth rate and phenylproponoid glycoside (PPG) production (a vital class of herbivore resistance phytochemicals) in variable populations of *M. guttatus* across an altitudinal gradient. Paired with evidence that coastal perennial populations generally produce higher relative concentrations of PPGs (Holeski *et al*. 2013), this could implicate the role of differential herbivore pressure as a biotic agent influencing divergent selection. Additionally, coastal populations have also been found to be adapted to oceanic salt spray (Lowry *et al*. 2008, 2009), a ubiquitous abiotic stressor in headland habitats.

Here, we manipulated a reciprocal transplant experiment to investigate whether the careful alteration of a region's selective agents can restore fitness in maladapted ecotypes. Exclosures can buffer plants from the detrimental effects of herbivory, salt spray, wind, and adverse temperatures while holding edaphic characteristics constant. To narrow the list of candidate selective agents affecting fitness in the field, plots at both coast and inland sites were protected with agrofabric exclosures. We then evaluated the fitness of both ecotypes at the end of the growing season, comparing relative performance of exclosure replicates to their controlled counterparts. This design allowed us to demonstrate that edaphic conditions are not among those agents selecting against inland *M. guttatus* recruits at the coastal extreme of California's coast-inland moisture gradient. Rather, some combination of aboveground agents, including vegetative herbivory and salt exposure, are instead most crucial in maintaining local adaptation at in coastal habitats.

MATERIALS AND METHODS

Genotype selection and growth conditions

To determine what combination of selective agents contribute to differential performance in divergent *M. guttatus* genotypes along California's coast-inland moisture gradient, we conducted a manipulative reciprocal transplant experiment. To verify that these effects are replicable within ecotypes, we used accessions from two coastal perennial (SWB and MRR) and two inland annual populations (LMC and OCC). All seeds used were derived from a single field mother per accession. LMC and SWB were collected in Mendocino County, CA and have been shown to be locally adapted through previous reciprocal transplant experiments (Lowry *et al*. 2008; 2010; Table S1). OCC and MRR were collected from Sonoma County, CA and have not been previously utilized in field reciprocal transplant experiments (Table 1). Seeds from each accession were gathered from the wild in previous years and stored in the Lowry Lab at Michigan State University (MSU). All accessions were grown at least one generation at the MSU greenhouse facilities to control for potential maternal effects and bulk seed stores. Since seed bulking can result in multiple generations of inbreeding in cataloged accessions, we chose among those inbred less than five generations (Table 1). Though some inbreeding depression was unavoidable, this screening allowed us to confidently negate the worst effects from our study.

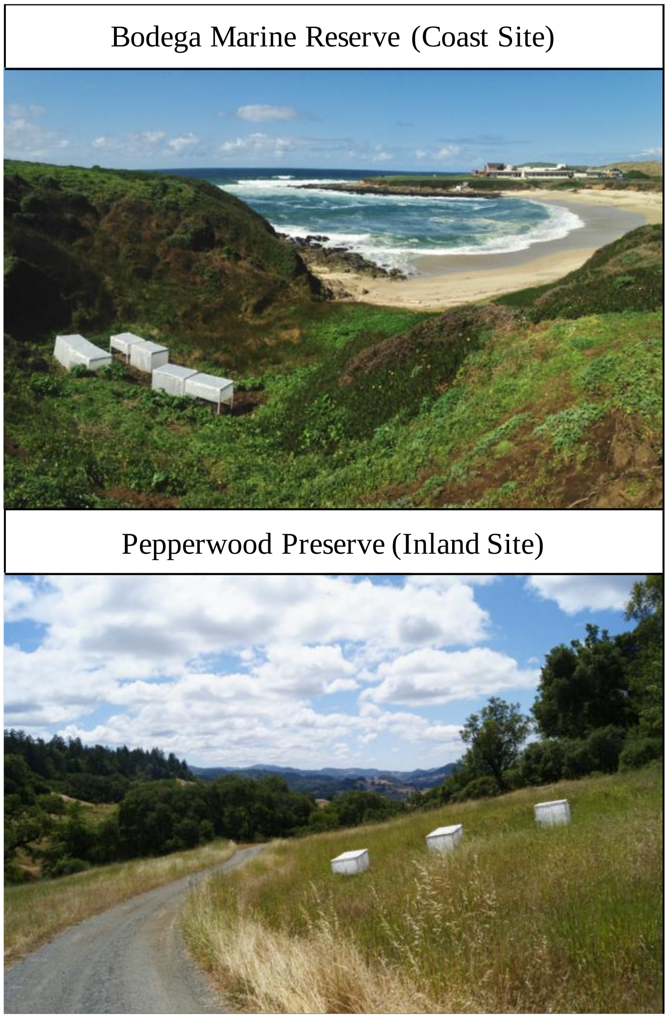
Table 1: Coordinates and inbreeding information of accessions used in this study.

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

Seeds were sown at UC Berkeley's greenhouse facilities on February 1st 2017. Each accession was sown as a lawn upon corresponding potting flats (54.28 cm L x 27.94 cm W x 6.20 cm H) filled with Sun Gro Horticulture's Sunshine Mix #1 (two trays per accession, eight in total), moistened prior to sowing with deionized (DI) water. Several hundred seeds were sown to satisfy the high replicate requirement necessary for our analyses and to ensure enough germinated for the experiment. Each of the resulting eight flats were subsequently misted with DI water and stored in a cold room at 4°C to stratify. Coastal flats were relocated to the greenhouse after 10 days of stratification, while their inland counterparts remained for an additional week (17 days). Considering the rapid pace at which inland annuals mature relative to coastal perennials, staggering their relocation allowed us to align the life stages in all our genotypes – regardless of ecotype – for planting in the field. Seedlings were germinated under constant conditions, misted daily, and exposed to 16 hours of daylight. All flats were transported from UC Berkeley's greenhouse facilities to the greenhouses at UC Davis' Bodega Marine Laboratory & Reserve on February 28th 2017.

Reciprocal transplant design

To test whether site-specific selective agents select against non-native ecotypes, a reciprocal transplant common garden experiment was planted along California's coast-inland moisture gradient. Ideal coast and inland sites were selected among two of Sonoma County's local ecological preserves, exhibiting little relative latitudinal deviation. Both gardens were planted among seeps inhabited by native populations of *M. guttatus*, demonstrating favorable conditions for species proliferation. The coastal garden was planted along a perennial seep at the southern end of Horseshoe Cove (Latitude: 38.315716°, Longitude: -123.068625°; 60.75 m from the ocean) on land managed by the UC Davis Bodega Marine Reserve (BMR) in Bodega Bay, CA (Figure 1). Our inland site was located along the margins of an ephemeral hillside seep (Latitude: 38.575545°, Longitude: -122.700851°; 39.84 km from the ocean) at the Pepperwood Preserve in Santa Rosa, CA. We established three split-plots at each field site.



Bodega Marine Reserve (Coast Site)

Pepperwood Preserve (Inland Site)

Figure 1: Photos of reciprocal transplant field sites in Sonoma County, CA. The coast garden is located upon a perennial seep running from the bluffs overlooking Horseshoe Cove, down into the sandy shoreline just short of the Pacific Ocean. The inland garden is planted along the margins of a seasonal seep in the Mayacamas Mountains of the Northern Inner Coast Ranges.

All six plots were established with the following dimensions: 216 cm L x 84 cm W (Figure 2B). Plots were positioned haphazardly no farther than 2 meters apart, leaving sufficient room for data collection and plot upkeep. Each plot was cleared of native vegetation prior to transplantation, simulating an artificial landslide event not unlike those common of California's headland bluffs (Collins and Sitar, 2008). All plots were subdivided into two 108 cm L x 84 cm W subplots (6 per site, 12 total) and randomly assigned a treatment (exclosure or shade control). Every plot was designed as a complete block, each consisting of one exclosure and one shade control subplot so that all genotypes underwent each treatment under every plot microclimate.

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 bolting. 25 replicates per population were planted at random in a grid within each designated subplot (100 per subplot, 200 per plot, 600 per site, and 1200 total). In all, 26 individuals died within a week of the initial planting (primarily inland replicates at the inland site) and were replaced immediately. All losses within the first week of planting were considered a result of transplant shock, thus justifying swift replacement. Any attrition thereafter was considered data.

Environmental manipulations

To test the combinatorial effects of coastal conditions on population mean fitness, we constructed multiple exclosures with the intent of excluding the variable aboveground stressors unique to each site – including transient herbivores and oceanic salt spray. 3 replicate exclosures were installed at each site (6 total) after planting, with dimensions complementing each subplot (108 cm L x 84 cm W x 87 cm H; Figure 2A). Remaining subplots were fashioned with a shade control variant designed to allow stressors access to control plants, yet replicate the same reduction in light transmittance experienced within exclosures.

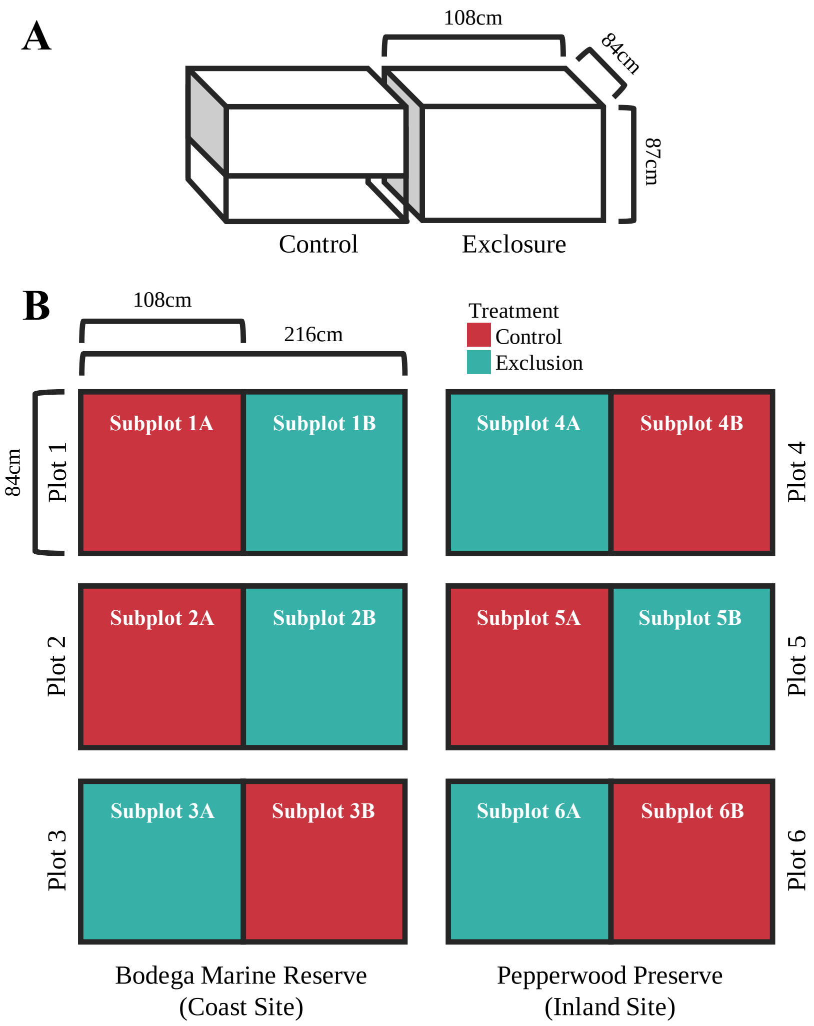


Figure 2: Exclosure schematics and experimental design. Depicted here are representations of both shade control shelters and exclosures used in this study with their appropriate dimensions (A), and a diagram of the split-plot design employed at both coast and inland sites (B). All shelters were fastened by zip tying two corners of each structure to complementary rebar stakes.

PVC pipes were used to construct the scaffold of each shelter, consisting of both a ground level and waist high rectangular quadrat attached at each corner via 4 PVC legs. All joints were reinforced with PVC cement to improve overall rigidity in order to withstand wind. A third quadrat was mounted with door hinges on the topside of the scaffold to be used as a lid for plot upkeep and data collection. To ensure that biological replicates were sufficiently buffered from the elements, the lid and scaffold of all exclosures were enclosed using medium weight agrofabric – ordered from OBC Northwest, Inc. (Pro-34 1.0 oz./sq. yd. with 70% light transmission). Agrofabric was also applied to the top two thirds of the shade control shelters, leaving the bottom open to the environment. All holes made in the agrofabric as a result of the zip ties used to fasten them were reinforced with clear repair tape. Lids were sealed to prevent aboveground herbivore intrusion by installing industrial strength VELCRO brand tape strips along the lip of each exclosure. We buried the exclosures in the ground from 8 - 13 cm, depending on location, to limit herbivore entry through the soil.

Effects of field manipulations on fitness

Due to concerns by Preserve staff about the potential for introgression of nonnative genes into the local historic gene pool, 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, the literature on local adaptation in the coast-inland / perennial-annual system demonstrates that the forces of selection are strong enough for survival to be a sufficient fitness measurement in coastal habitats. Thus, to study the effects of habitat and exclosure on the performance of both ecotypes, we collected survival data at seven time points throughout the growing season. All aboveground *M. guttatus* vegetation (dead or alive) was harvested at the end of the project and transported in brown paper bags (June 13th – 15th, 2017). These samples were shipped to Michigan State University where they were dried in an oven at 60°C for 2 weeks. Each sample was weighed with an analytical balance to quantify dry aboveground biomass, a valuable but imperfect indicator of plant performance and fecundity (Younginger *et al.* 2017).

To test for differential exposure of aerosolized salt spray across treatments at the coast, salt traps were designed and installed among all plots roughly following the instructions of Yura (1997) & Yura and Ogura (2006). In total, 6 salt traps were deployed at the coast site (one per subplot) and 2 traps at the inland site (one per treatment). Traps consisted of a four-sided rectangular plastic prism fashioned upon a small cylindrical post. Clear vinyl badge holders were clipped upon each face (one per cardinal direction) to act as protective sheaths for Whatman Brand #2 Qualitative Medium filter paper inserts (cut to size: 8.6 cm L x 5.8 cm W). Badge holders have a native cutout to allow the filter paper to absorb all incident salt spray. Traps were left to collect salt for two weeks starting on May 7th, 2017 (24 coastal / 8 inland inserts) and were eventually sealed in individual ziplock bags, labelled, and transported back to Michigan State University. Inserts were left to air-dry for 24 hours, placed in Erlenmeyer flasks (1 per trap, 8 total) to soak in 50 ml ultrapure water, and shaken for 1 hour to extract salts. Samples were filtered of all resulting fibers and debris with Whatman No. 44 filter paper and subsequently analyzed for Sodium (Na) using inductively coupled plasma optical emission spectrometry (ICP-OES; Olesik 1991).

To quantify the degree to which our exclosures affected herbivore activity, we kept a detailed record of how many replicates had experienced any evident herbivore damage throughout the extent of our experiment. At the end of the season, all plants were categorized as having experienced herbivory or being unscathed by herbivores. Any replicate that died prior to the accumulation of any obvious herbivore related injuries were ultimately considered 'unscathed.' We ran a general linear model (GLM) using a binomial distribution to analyze herbivory in the context of presence / absence. Separate models were run for each site. No random effects were evaluated here as the goal was to determine whether the exclosure treatment had indeed effectively reduced the incidence of herbivory at either site.

Analysis of transplant data

To confirm whether some combination of aboveground stressors contribute to fitness across transplant sites, our data were analyzed using an ASTER modeling approach (Geyer *et al*. 2007; Shaw *et al.* 2008). ASTER is a module developed for the statistical program R that provides a powerful tool for scrutinizing multiple fitness measures in a single analysis, accounting for order (i.e. flower, to fruit, to seed) and their individual probability distributions. The power of ASTER lies in its ability to calculate an expected fitness value for all biological replicates given the interdependence of each fitness measure. We used ASTER to analyze a composite of 8 fitness components: survival to weeks 1 – 7, all modeled as Bernoulli (0 or 1), and the total biomass accrued, here modeled as a normal distribution. Due to coding constraints in ASTER, any replicates with a non-zero mass that died before the final observation date (measured post mortem) had their biomass values altered to zero (52 of 1200 observations were affected, each with biomass values that deviated little from zero). Likelihood ratio tests were constructed by comparing nested null models to test alternative hypotheses.

We also employed a generalized linear mixed modeling approach (GLMM) to further analyze these data. The ASTER module cannot account for random effects, thus running GLMM aided us in confirming our ASTER results. Site specific models were developed in lieu of a more comprehensive model, as we were less interested in the effects of site on fitness, but rather the effects of treatment dependent on site. Response variables included were survival to harvest (week 7), modeled using a binomial distribution, and total biomass accrued. Since there is no proper modeling distribution for continuous zero-inflated data in the GLMM framework (data ASTER is quite useful at dealing with), we treated all zero biomass values as missing data and removed them from the analysis. The remainder was modeled using a gamma distribution. The following GLMM scaffold was developed for all combinations of site and response (4 models in total): Response ~ Treatment + Ecotype + Treatment:Ecotype + (1|Plot) + (1|Accession). While both ASTER and GLMM modeling approaches have limitations, they complement each other by providing a function the other cannot.

RESULTS

General patterns

Overall, 89% of transplants survived to harvest at Pepperwood (inland site) and 73% survived at Bodega Bay (coast site). Exclosures and control subplots at the inland site saw comparable survival rates, with 90% in the former and 88% in the latter. However, survival diverged markedly at the coast with 97% survival in exclosures and 49% in shade control subplots. This vast disparity is driven mostly by discrepancies in ecotype performance dependent on subplot treatment. The inland site generally produced transplants with limited dry biomass, exhibiting an average of about 0.0748g. In contrast, the coast site experienced robust growth all around, ending the season with an average biomass of near 1.16g.

Fitness effects at the coastal field site

There were striking differences in the responses of coastal and inland accessions to the exclosure treatment at the coastal field site. At the coast, 87% fewer inland control replicates survived until the end of the experiment than the coastal control plants. The few inland individuals that did survive amassed little in terms of vegetative biomass (Fig. 3A/B). In contrast, the inland plants within exclosures experienced nearly the same survival rates and accrued biomass at similar levels as coastal replicates under either treatment (Fig. 3A/B). Our GLMM approach demonstrated a significance of ecotype x treatment interaction at the coast field site ($P < 0.0001$; Table 2), confirming that differential fitness performance between ecotypes was dependent on treatment. The biomass model showed the same effect and directionality of ecotype x treatment interactions, though the magnitude of this effect was lesser ($P < 0.03982$; Table 2).

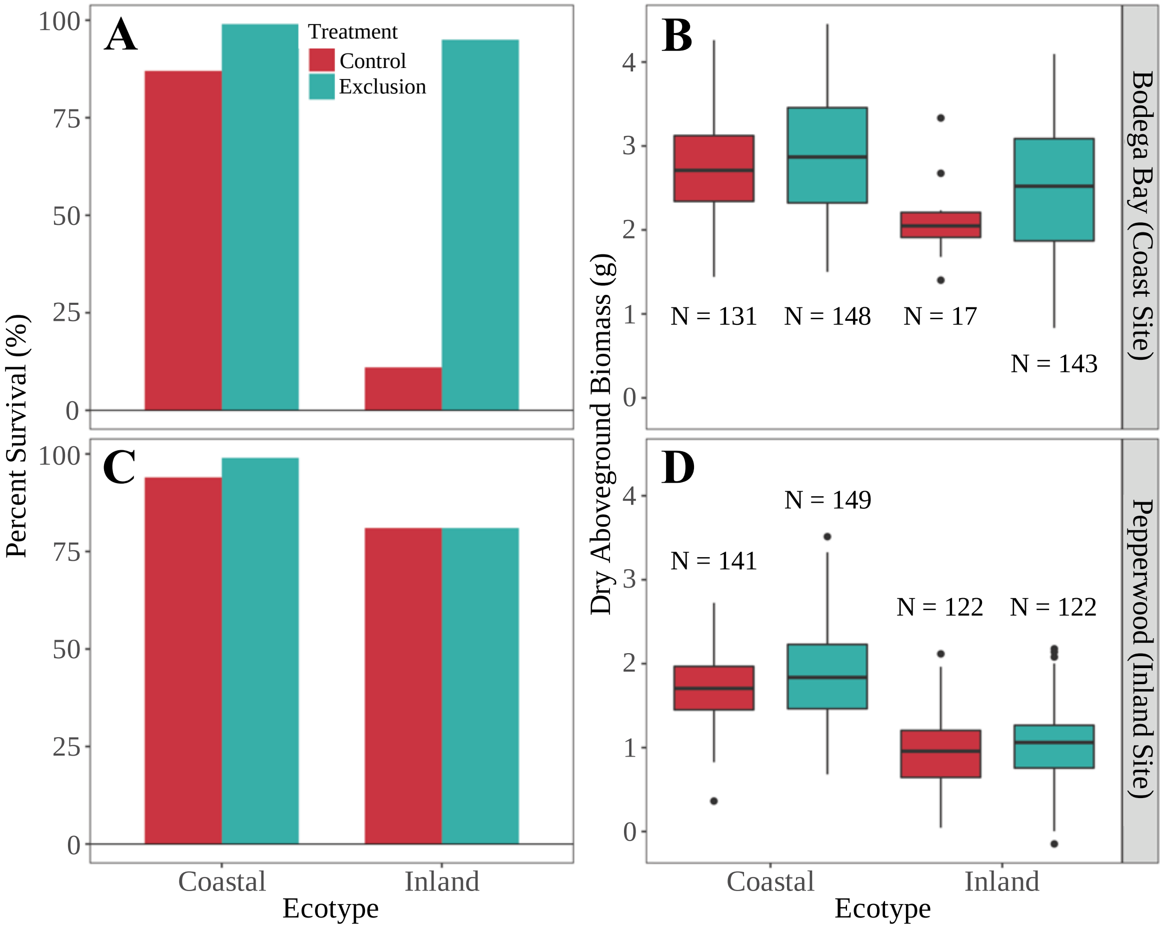


Figure 3: Observed survival and dry aboveground biomass in the field. Fitness proxies in all replicates across both reciprocal transplant gardens were measured to model the effect of ecotype, treatment, and ecotype x treatment interactions on in situ performance. Percent Survival is displayed in bar plots specific to the coast (A) and inland (C) sites. Box and whisker plots demonstrate dry aboveground biomass at the coast (B) and inland (D) sites in only those replicates that had a non-zero mass at harvest (N = adjusted non-zero sample size).

ASTER modeling generally confirmed the patterns found by GLMM analysis. Not only were there strong treatment effects at the coast (*P* < 0.0001; Table 3), but treatment was also found to significantly affect expected biomass for each ecotype (*P* < 0.0001; Table 3). Although the exclosure treatment led to an increase in the expected mean vegetative biomass of both ecotypes at the coast, the response of inland plants to the treatment was much more dramatic. This is made most evident in Figure 4A, where inland exclosure replicates are shown to have accrued an expected mean biomass of 1.58g while their control homologs were calculated at -1.33g.

Table 2: Analysis of fitness using a Generalized Linear Mixed Model (GLMM). Response ~ Treatment + Ecotype + Treatment:Ecotype + (1|Plot) + (1|Accession). This modeling scaffold was run for all combinations of site and response variable. The appropriate p values for our main effects and subsequent interaction term are provided below. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

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*

Table 3: Analysis of fitness using an ASTER-based modeling approach. Our ASTER models analyzed a composite of eight fitness components, including survival from weeks 1 – 7 and a post-harvest measure of dry aboveground biomass. These components were aligned in the following directional graph in order of general causality: survival to week 1 → survival to week 2 → survival to week 3 → survival to week 4 → survival to week 5 → survival to week 6 → survival to week 7 → biomass accrued. All factors were tested by likelihood ratio tests using nested null models. ***$P<0.001$.

Site	Factor Tested	Null df	Alt. 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***
	Interaction	10	11	-930.70	-903.31	1	27.385	<0.0001***
inland	Treatment	9	10	-541.45	-539.24	1	2.205	0.1376
	Interaction	10	11	-539.24	-503.36	1	35.887	<0.0001***

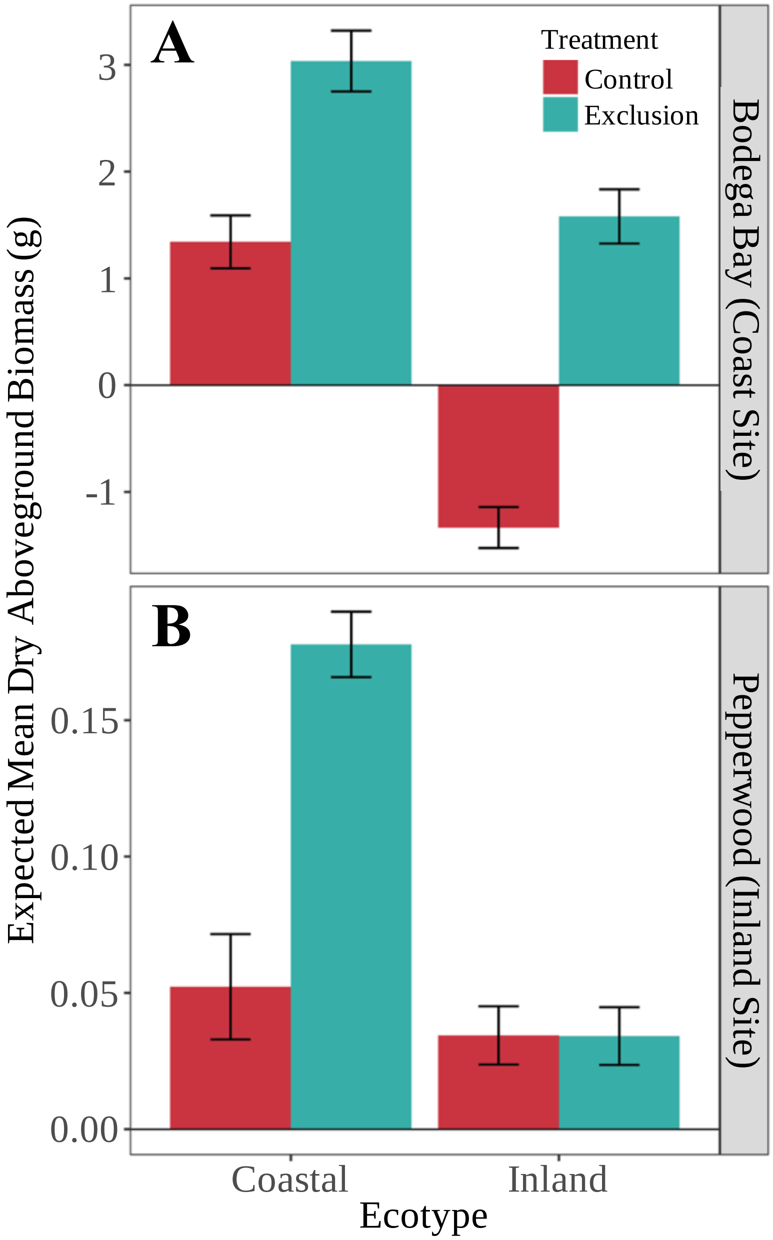


Figure 4: Expected mean dry aboveground biomass. Reported separately here by coast (A) and inland (B) sites. Here, expected biomass represents the ultimate measure of Darwinian fitness (specific to this study) in each replicate under all treatments by accounting for survival in combination with dry aboveground biomass using ASTER modeling. 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.

Fitness effects at the inland field site

Ecotype, treatment, and ecotype x treatment interaction all had a significant effect on survival and biomass at the inland site ($P < 0.0001$) – with the exception of treatment's effect on biomass (Table 3). However, the magnitude of these differences was relatively small, with a 19% difference in survival between ecotypes and a 3% difference in survival between treatments. The pattern driving divergent performance at our inland site was primarily due to ecotype, where its effects on survival ($P = 0.0015$; Table 3) and biomass ($P < 0.0001$; Table 3) were consistently and markedly significant relative to treatment. Coastal replicates both survived in greater numbers and produced more vegetative biomass than their inland counterparts (Fig. 3C/D). Regardless of these differences, most replicates at this site still survived to harvest.

There was no significant effect of treatment in our ASTER models ($P = 0.1376$; Table 4). However, the ecotype x treatment interactions was significant for biomass ($P < 0.0001$; Table 4). This interaction is the result of an almost three-fold difference in expected biomass between treatments, with exclosure biomass eclipsing that of our control plots (Figure 4B).

Quantifying salt spray and herbivory

Quantifying the concentration of incident Na allowed us to scrutinized whether our exclosures had any significant effect on incident salt levels at the subplot level. Na concentrations were found to be elevated in the ambient conditions of control subplots at the coast in comparison to any other combination of treatment and site (Figure 5). Salt samples collected from coast control subplots demonstrated a near consistently two-fold higher level of Na over those sheltered within exclosures. The inland plot was confirmed to show no remarkable difference in Na concentration across treatments. Furthermore, both inland samples were demonstrably similar in Na levels to those of our coast exclosures. Analysis of a 2% nitric acid blank and clean filter control revealed that our solvent and filter slips had small-to-negligible effects on the Na content of our test samples. This indeed verifies a pattern of ambient salt reduction as a consequence of our exclosure installations.

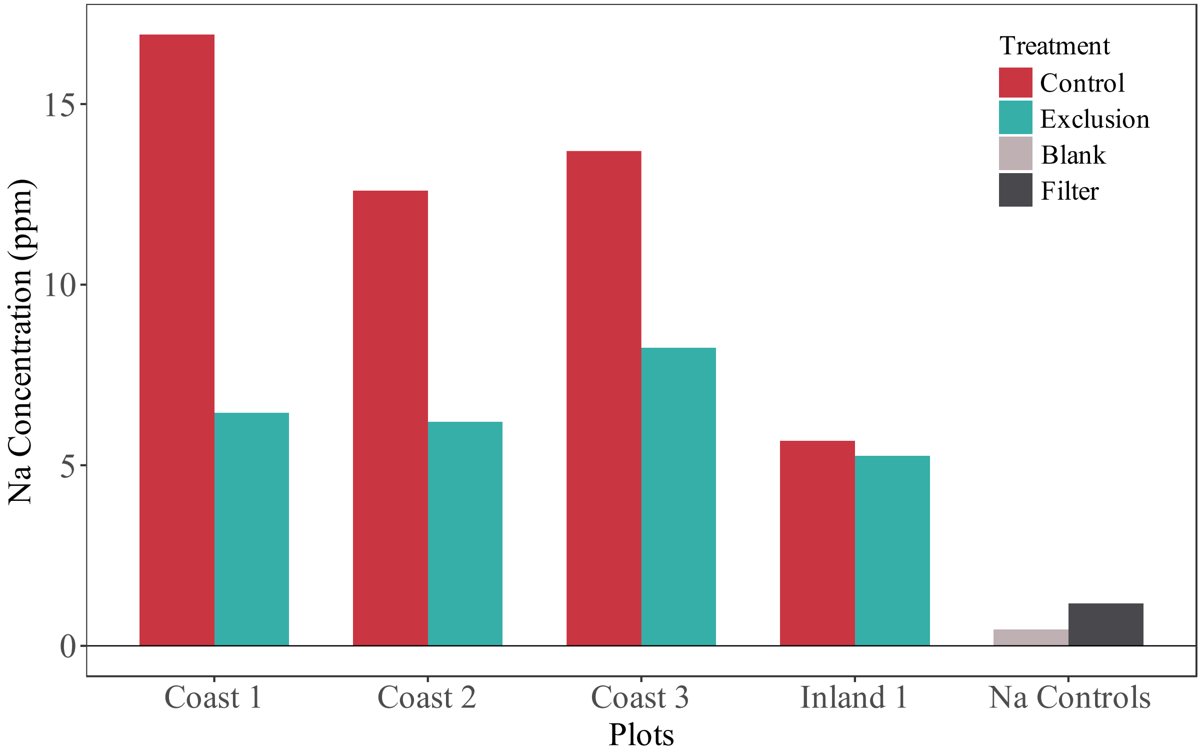


Figure 5: Elemental Sodium (Na) concentrations in ppm. All salt traps were left in the field for two weeks and analyzed for Na using inductively coupled plasma optical emission spectrometry (ICP-OES). Control samples were subject to elements native to each site and plot microhabitat. Exclusion samples 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 coast plots and one of three total inland plots are represented in the data above

Similar to our Na analyses, quantifying the incidence of herbivory provided valuable insight on the effectiveness of our exclosures. Treatment had a significant effect on herbivory at the coast site ($P < 0.0001$), nearly eliminating the incidence of herbivore damage to the plants within the exclosures (Table 4). Ecotype nor ecotype x treatment interactions were found to affect herbivory at the coast ($P = 1$). Though nearly a quarter of coastal and inland controls experienced some herbivore damage at the coast site, there were few observed instances of herbivory at the inland site regardless of treatment (Table 4). In contrast to the coast site, there was no evident effect of treatment on the incidence of herbivory at the inland site ($P = 0.8105$). Only ecotype had a significant effect on rate of herbivore damage ($P = 0.0189$), although this difference was relatively small (Table 4). The ecotype x treatment interaction was also not found to be significant at the inland site ($P = 0.8104$).

Table 4: A report of herbivore related incidences across all transplanted replicates. % Herbivorized was calculated by dividing the final number of predated plants by the total present in each treatment group (150 for each of the 8 combinations of Site, Ecotype, and Treatment).

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

Our results confirm that some combination of aboveground stressors, including salt spray and herbivory, contributes to the adaptive divergence of coastal perennial and inland annual ecotypes of the Seep Monkeyflower, *M. guttatus*. While coastal conditions strongly favor the persistence of native genotypes, nonnative inland accessions at the coast were shown to be rescued by exclosure treatments. The effect was so strong that fitness was not merely restored, they thrived at levels comparable to their coast adapted counterparts. While evidence demonstrates that the evolution of annual populations along California's ephemeral seeps and streams may primarily be driven by seasonal drought (Vickery 1952; Lowry *et al*. 2008), coastal denizens, in contrast, have adapted to withstand the adverse effects of prolonged salt exposure and, potentially, a suite of hostile native herbivores – though the mechanisms of which remain unresolved.

Patterns of local adaptation

For the conditions of local adaptation to be met, a population or collection of populations native to one region of a species' range are thought to trade off their ability to thrive elsewhere in an effort to prioritize adaptation to their home habitats (Kawecki & Ebert 2004; Hereford 2009). Tradeoffs associated with local adaptation may occur as consequence of a specialized adaptive trait's general inability to remain beneficial under every environmental regime (VanWallendael *et al. In review*). Not in keeping with previous findings on this system, survival of both native and non-native genotypes at the inland site remained comparable throughout the entire growing season. While this result appears to be at odds with local adaptation, it was entirely expected. We have shown in previous studies that despite the consistent performance of coastal transplants early in the growing season at inland field sites, nearly all are killed by the summer drought before they have the opportunity to flower (Hall et al. 2006; Lowry et al. 2008; Hall et al. 2010; Lowry & Willis 2010). In contrast, fast growing inland annuals survive to flower at very high rates at inland field sites. Thus by ending the experiment early on before the summer drought can properly affect fitness, our data did not capture selection imposed by drought, a phenomenon well characterized in previous studies.

The most significant result of our study is the striking effect of the exclosure on survival and biomass of inland annual transplants at the coastal field site, despite sharing the edaphic conditions of neighboring control replicates. These exclosures essentially freed non-native transplants of the detrimental effects of natural selection at the coastal field site. In contrast, exclosures did not have as great an effect at the inland field site, where coastal perennial plants survived at similar levels to inland annuals. Our analyses demonstrate that a simple control of various stressors in the field can overcome the environmental variables that limit inland fitness at the coastal field site. Thus, some suite of aboveground agents perform the bulk of selection that maintains local adaptation in coastal populations of *M. guttatus*.

The effect of our treatment at the inland field site was unexpected, since hypothesized agents of selection like salt spray are not a factor in this habitat and rates of herbivory are much lower. The elevated biomass of the coastal perennial transplants within exclosure were most exaggerated in two of the three inland plots, coinciding with exclosure subplots planted closer to the interior of the seep where soil moisture levels remains favorable for longer. While this result could be an artifact of experimental setup, the same trend was not evident among inland replicates. Therefore, it may be that an agent like herbivory plays some partial role in the performance of coastal transplants in inland habitats. Future studies conducting detailed quantification of herbivore damage will be needed to make further conclusions.

Salt spray and herbivory as agents of selection

Salt stress, whether derived from topical incidence or root uptake, can have a range of adverse effects on plant osmotic homeostasis (Boyce 1954; Humphreys 1982; Griffiths 2006). Every aspect from a plant's physiology to its metabolism can experience abnormal or even fatal defects in the presence of high environmental salt concentrations (Boyce 1954; Humphreys 1982; Griffiths 2006). However, coastal populations of *M. guttatus* – often occurring within a few meters of the wavebreak – are known to be tolerant of topical salt application (Lowry *et al*. 2008, 2009). This remains a putative adaptation to high ambient salt conditions along the coast. The majority of inland replicates at Bodega Bay experienced complete vegetative loss (with no remaining tissue left to scrutinize), not inconsistent with early salt-induced decay. Few inland controls persisted long enough to successfully form buds. However, among all bolting survivors, floral stalks and lateral branches turned brown and produced no healthy flowers, likely succumbing to salt exposure. Similar instances of premature vegetative senescence were noted in inland replicates within coastal exclosures, but only limited to those tissues in direct contact with the agrofabric. This vegetation was presumably experiencing advance stages of salt stress as a consequence of oceanic salt accumulation on exclosure barriers. Corroborated by our fitness data, these observations demonstrate high salt susceptibility in inland *M. guttatus* genotypes at the coast. These results are consistent with laboratory experiments that have shown differences in salt tolerance between coastal and inland populations of *M. guttatus* (Lowry *et al.* 2008; 2009).

Pervasive herbivore pressure can lead to the evolution of specialized adaptive traits. These mitigation techniques can be broadly categorized as either imparting some degree of tolerance or resistance towards potential predation events (Strauss and Agrawal 1999; Núñez-Farfán *et al.* 2007). This study was in part designed to scrutinize whether local adaptation in a coast/inland, perennial/annual context led to divergence in herbivore tolerance – that is, maintained fitness despite predation. Since the damage metrics collected at each site were few, it remains difficult to parse the effects of salt stress and herbivory on fitness across ecotype, treatment, and site. Field observations did reveal significantly higher rates of herbivory among control replicates of all genotypes at Bodega Bay, demonstrating the exclusionary power of our agrofabric treatments. Yet there appeared to be no apparent bias towards one ecotype over another. However, contrary to our predictions, mammalian predation by resident California Voles (*Microtus californicus*) made up a significant portion of damage done to control replicates at the coast – most often evidenced by the complete removal of floral stalks and branches.

Though coastal controls experienced more herbivory than their exclosed counterparts, it did not appear ubiquitous enough to be the sole explanation for such striking fitness differentials across treatments. While the lower biomass acquisition of coastal control replicates can be attributed to some loss of tissues via predation, most remained rosettes, exhibiting a slower growth rate regardless of herbivore damage, ultimately driving average mass down among control replicates. Unique to coastal controls was an almost uniform degree of elevated anthocyanin production in localized tissues. This demonstrates that despite adaptations to thrive in adverse salt conditions, coastal plants are not completely tolerant of oceanic salt sprays. Protection from these stressors enabled coastal exclosure replicates to grow more robustly and begin bolting before the end of the experiment. Though salt is likely the predominant agent of selection at the coastal extent of *M. guttatus*'s habitable range, it is difficult to attribute this stress to early mortality of inland control replicates. It could be equally possible that they were consumed in their entirety not long after their initial planting, leaving no clear indication of herbivory. Though some combination of herbivory and salt spray have clearly contributed to local adaptation among coastal populations of *M. guttatus*, we cannot parse their relative importance without further manipulative experiments.

Hypothesized mechanisms of divergent selection

Recent population genomic analyses by Gould *et al.* (2017) discovered strong sequence divergence between coastal perennial and inland annual populations of *M. guttatus* for multiple genes putatively involved in hormone-mediated plant growth responses. Corroborated by a recent understanding of the perennial ecotype's more robust defensive phytochemical makeup (Holeski *et al.* 2013), we have grown more confident in postulating the existence of a critical growth-defense tradeoff in this system. However, our findings provide no conclusive evidence of covariation between defense and herbivore pressure across California's coast-inland moisture gradient. Evident above all is the importance of variation in the rate of development as a consequence of local adaptation to regions of variable growing season lengths, similar to the argument made by Kooyers *et al.* (2017). Within the context of the resource allocation hypothesis, greater defensive capabilities among the coastal *M. guttatus* ecotype may simply be the result of more favorable yearlong growing conditions, freeing them to focus some resources on defense rather than primarily on growth and reproduction. Alternatively, it could be because slower growing vegetation is more valuable (Mason and Donovan 2015), thus a greater focus is placed on their defense than that of an annual with faster vegetative turnover. Further elucidation of the mechanisms responsible for local adaptation in this system will require a combination of molecular and field experiments.

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