Plant hormone manipulation impacts salt spray tolerance, which preempts herbivory as a driver of local adaptation in the yellow monkeyflower, *Mimulus guttatus*

Running head: Local adaptation to salt spray

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Abstract

A major challenge in evolutionary biology is identifying the selective agents and phenotypes underlying local adaptation. Local adaptation along environmental gradients may be driven by trade-offs in allocation to reproduction, growth, and herbivore resistance. To identify environmental agents of selection and their phenotypic targets, we performed a manipulative field reciprocal transplant experiment with coastal perennial and inland annual ecotypes of the common yellow monkeyflower (*Mimulus guttatus*). We manipulated herbivory with exclosures built in the field and exogenously manipulated hormones to shift allocation of plant resources among growth, reproduction, and herbivore resistance. Our hormone treatments influenced allocation to reproduction and phytochemical defense, but this shift was small relative to ecotype differences in allocation. Herbivore exclosures reduced herbivory and increased fitness of plants at the coastal site. However, this reduction in herbivory did not decrease the homesite advantage of coastal perennials. Unexpectedly, we found that the application of exogenous gibberellin increased mortality due to salt spray at the coastal site for both ecotypes. Our results suggest that divergence in salt spray tolerance, potentially mediated by ecotype differences in gibberellin synthesis or bioactivity, is a strong driver of local adaptation and preempts any impacts of herbivory in coastal habitats that experience salt spray.

Key words: local adaptation, monkeyflower, herbivory, salt spray, gibberellin, *Erythranthe guttata*

Introduction

Organisms experience dramatically different environmental conditions throughout their geographic ranges. Spatial gradients in abiotic factors, such as temperature, salinity, and water availability, as well as biotic factors, such as the presence of competitors, predators, and mutualists, can generate divergent natural selection (Kawecki & Ebert, 2004; Maron et al., 2014). This divergent selection can in turn lead to evolutionary responses in traits that increase fitness in local environments, and result in the evolution of local adaptation (Clausen et al., 1940; Hereford, 2009; Kawecki & Ebert, 2004; Leimu & Fischer, 2008; Wadgymar et al., 2022). Identifying the causal environmental factors contributing to adaptation is a major challenge because environmental conditions often co-vary and thus, experimental manipulations are necessary to identify the environmental agents of selection (Briscoe Runquist et al., 2020; Hargreaves et al., 2020). Likewise, the phenotypic targets of selection are challenging to identify because traits are often highly correlated, so approaches that minimize trait correlations (e.g., using hybrids) or manipulate trait variation independently of other traits are necessary to identify adaptive traits (Wadgymar et al., 2017, 2022). Despite their importance, experiments that simultaneously manipulate putative environmental selective agents and their phenotypic targets are uncommon (Wadgymar et al., 2017, 2022). In this study, we isolate the effect of a putative selective agent, herbivory, at sites that vary in two abiotic factors, salt spray and soil moisture, and manipulate trait variation using hormone applications to identify the environmental and biotic drivers of local adaptation.

Traits that increase fitness on one end of an environmental gradient can reduce fitness on the opposite end of that gradient, resulting in fitness trade-offs (Kawecki & Ebert, 2004). Trade-offs are often caused by evolutionary changes in the allocation of limited resources to critical biological functions, including growth, reproduction, and defense (Bazzaz et al., 1987; Herms & Mattson, 1992). Theory predicts that resource allocation to herbivore defense should depend on the risk and consequences of herbivory on fitness, and models of the evolution of plant defense assume a cost to the production of herbivore defenses (Rhoades, 1979; Stamp, 2003). Within species, allocation to herbivore resistance frequently trades-off with allocation to reproduction (Agren & Schemske, 1993; Cipollini et al., 2017; Heil & Baldwin, 2002; Stowe & Marquis, 2011; Strauss et al., 2002), and increased allocation to herbivore resistance is associated with longer growing seasons. This association could be driven by multiple factors, including a longer period of vegetative growth and resultant longer exposure risk and apparency to herbivores and/or greater herbivore pressure (Feeny, 1976; Hahn & Maron, 2016; Kooyers et al., 2017; Mason & Donovan, 2015; Smilanich et al., 2016).

The physiology underlying potential trade-offs is still unclear but is likely due to the evolution of plant hormone pathways in response to different environmental conditions. Recent studies have shown that shifts in the allocation of resources from rapid growth to herbivore resistance are made through a set of interacting gene networks (Aerts et al., 2021; Campos et al., 2016; Havko et al., 2016; Huot et al., 2014; Kazan & Manners, 2012; Monson et al., 2022). Jasmonates (JA) are key regulatory hormones involved in the response of plants to herbivore attack (Havko et al., 2016; Zhang & Turner, 2008). While JA production increases herbivore defense, it also can inhibit rapid plant growth through interactions with other gene networks (Kazan & Manners, 2012; Yan et al., 2007; Yang et al., 2012; Zhang & Turner, 2008). For example, the interactions of JAZ (Jasmonate ZIM-domain) genes with DELLA genes in the signaling pathway of Gibberellin (GA) growth hormones are thought to play a key role in mediating resource allocation (Havko et al., 2016; Hou et al., 2013; Yang et al., 2012). However, evidence that evolutionary changes in the GA pathway lead to changes in the relative allocation of resources to rapid reproduction, long-term growth, and herbivore resistance is still lacking. Further, no study that we are aware of has evaluated the physiological mechanisms underlying the evolution of intraspecific trade-offs driven by allocation to growth, reproduction, and defense that occurs when natural populations adapt to different habitats. Furthermore, phenotypic changes induced by the exogenous application of hormones allow a powerful test linking phenotype to fitness across habitats in carefully controlled field studies.

An excellent system for investigating the mechanisms responsible for the evolution of adaptive trade-offs in growth, reproduction, and resistance are locally adapted ecotypes of the yellow monkeyflower, *Mimulus guttatus* (syn. *Erythranthe guttata*). Previous reciprocal transplant experiments showed that the primary environmental factor contributing to local adaptation at inland sites was the onset of summer drought (Hall & Willis, 2006; Lowry et al., 2008), while a combination of above ground factors, including salt spray and herbivory, contributed to adaptation in coastal habitats (Lowry et al., 2009; Popovic & Lowry, 2020). Inland populations of *M. guttatus* are typically small annuals that allocate resources primarily to reproduction in order to flower prior to the onset of summer drought. Coastal populations, which occur in habitats with year-round soil moisture, are large obligate perennials that allocate resources primarily to long-term growth (Baker et al., 2012; Baker & Diggle, 2011; Hall et al., 2010; Hall & Willis, 2006; Lowry et al., 2008). Coastal populations have higher levels of phytochemical defenses (phenylpropanoid glycosides, PPGs) and experience higher levels of herbivory than the inland annual populations (Holeski et al., 2010, 2013; Lowry et al., 2019). In the greenhouse, coastal populations are more responsive to exogenous applications of gibberellin (GA3) than annuals and respond by recapitulating the elongated growth habit of inland annual populations (Lowry et al., 2019). As a result, we hypothesize that natural variation in allocation to rapid reproduction, long-term growth and resistance is the result of molecular changes that alter the interactions of the gibberellin (GA) and jasmonic acid (JA) pathways.

In this study, we performed a manipulative reciprocal transplant experiment to test whether trade-offs between allocation to vegetative growth, reproduction, and herbivore resistance contribute to local adaptation at opposite ends of an environmental gradient. We predicted that increased allocation to reproduction (via early flowering) would increase perennial fitness at the inland site, where earlier flowering would rescue fitness for individuals that typically perish before the onset of summer drought, and thus decrease annual homesite advantage. We also expected that increased allocation to vegetative growth (via delayed flowering) and herbivore resistance would increase annual fitness at the coast, where we expected herbivore pressure to be higher. Finally, we predicted that reduction of herbivory via exclosures would rescue annual fitness on the coast, and thus decrease perennial home site advantage. While our study was designed to focus on the role of hormone manipulation on defense against herbivory, we instead discovered that our hormone manipulations had a much larger role in causing susceptibility to stress imposed by oceanic salt spray. This surprise discovery altered our approach to data analysis, which we describe below in the methods and results.

Materials and Methods

Study location

We performed a reciprocal transplant experiment at two sites – a coastal seep at the Bodega Marine Reserve in Bodega Bay, CA (Latitude: 38.3157, longitude: −123.0686), and an inland seep at the Pepperwood Preserve near Santa Rosa, CA (latitude: 38.5755, longitude: −122.7009).

Plant material

We used outbred maternal families from a coastal perennial population from Bodega Bay, CA (*n* = 5 families, BHW: 38.303783, -123.064483) and an inland annual population north of Sonoma, CA (*n* = 4 families, CAV: 38.342817, -122.4854). Outbred maternal families were generated by crossing field collected maternal families in the greenhouses at Michigan State University. Seeds from these outbred families were sent to UC Berkeley, planted, and placed in a 4°C cold room on January 27, 2020. We staggered perennial and annual germination to synchronize their development (following Popovic & Lowry, 2020). A week after beginning stratification, perennial seeds were moved into a 16-hr day length growth chamber for germination. Two weeks after beginning stratification, annual seeds were moved to the same 16-hr day length growth chamber. Seedlings were transported from UC Berkeley to the Bodega Marine Reserve (BMR) greenhouse on February 20, 2020, and then were transplanted seedlings into individual cell packs over the course of a week.

Hormone treatments

We altered the allocation phenotypes of each ecotype by manipulating hormone levels of plants with exogenous applications of gibberellin (GA3, a growth hormone), paclobutrazol (a GA inhibitor), and methyl jasmonate (a hormone that induces herbivore resistance and antagonizes GA) to test the role of those hormone pathways in adaptive trade-offs between rapid reproduction versus long-term investment in vegetative growth and herbivore resistance. Following a week of transplanting in the BMR greenhouse, we randomly assigned cell pack trays to one of three hormone treatments or control. We sprayed plants with a 100 µM solution of gibberellic acid (Consolidated Chemical Solvents LLC, following Lowry et al., 2019), 10 mM methyl jasmonate (TCI America, Portland, Oregon, USA), and 14.3 mg/L solution of paclobutrazol (General Hydroponics, Santa Rosa, California, USA). Concentrations of methyl jasmonate and paclobutrazol were chosen after conducting dose response experiments at the MSU greenhouses in winter 2019. These concentrations were chosen based on the minimum concentration needed to elicit a phenotypic change relative to controls without detrimental effects (e.g., leaf damage, stunting, death). Using a spray bottle, we sprayed individual plants 5 times, corresponding to 3.5 mL of solution. The control consisted of spraying plants with 3.5mL of a 0.25% ethanol solution since a dilute ethanol solution was needed to dissolve methyl jasmonate and all hormones were dissolved in a 0.25% ethanol solution. Hormones were applied once on a single day in the greenhouse prior to field transplanting. All trays were covered with clear plastic domes for 24 hours and moved to different benches to prevent cross contamination.

Field planting

Prior to transplanting, we removed vegetation from ten (108cm x 84cm) plots at each site. We dug trenches along the edge of each plot to bury the bottom of our control and exclosure structures. At the Pepperwood Preserve, we transplanted 800 seedlings on March 9, 2020, and 200 seedlings on March 12, 2020. At the Bodega Marine Reserve, we transplanted 800 seedlings on March 10, 2020, and 200 seedlings on March 13, 2020. Plants were fully randomized within each block (*n* = 100 seedlings/block) and labeled with a plastic tag.

Herbivore exclosures

To lessen the effect of herbivory and potentially measure a cost to defense production in the absence of herbivores, we deployed herbivory exclosures on four out of ten plots at each site (Figure 1). A previous reciprocal transplant experiment at our study sites used exclosures that blocked all above-ground factors using agrofabric (Popovic & Lowry, 2020), and thus could not separate the effects of salt spray and herbivory on plant fitness. Thus, we designed exclosures that excluded many herbivores but allowed salt spray to pass through. The exclosures were 108 cm long x 84 cm deep x 87 cm tall and constructed of 3/4" pvc pipe covered with fiberglass window-screen (18x16 mesh/inch) that was affixed with fishing line and marine epoxy. Each exclosure had screen doors along both long sides that were attached with velcro to allow access to the plots. The screen extended 4 inches down into the soil around the plots. To control for shading or moisture-collection due to the screen, the remaining six plots at each site were covered with control structures. These structures differed in that only the tops and 30 cm down each side were covered with window screen.

Field Censuses

After transplanting, we performed regular censuses of our transplant sites recording survival, the presence of herbivore damage, the identity of herbivores (when possible), the presence of salt-spray damage, and the presence and number of reproductive structures (buds, flowers, and fruits). In our census, we distinguished damage and death caused by salt spray from herbivory: salt-damaged leaves appeared necrotic and brown and exhibited no sign of herbivore damage (i.e., no missing tissue), when salt damage spread to the entire plant and no green tissue remained, we considered plants to be killed by salt spray. We were prevented from accessing our transplant sites for two weeks at Bodega Marine Reserve and seven weeks at Pepperwood Preserve after transplanting due to the 2020 COVID-19 pandemic lockdowns. Due to site differences in growing season length, and restricted access due to the 2020 COVID-19 pandemic, we censused each site at different intervals and for different lengths of time (Pepperwood Preserve (inland site):12 censuses over 139 days, Bodega Marine Reserve (coastal site): 22 censuses over 194 days). Since we were unable to access our sites for weeks because of the pandemic, we missed observing the first flower opening for many annual plants. For these plants, we estimated the onset of flowering as the date we first observed any reproductive structures. Our censuses occurred on a roughly weekly basis after we were able to re-access our sites and we continued to estimate the onset of flowering based on the initial observation of a bud, flower, or fruit during each census.

Plant chemistry

We sampled leaves for chemical analysis 55 to 57 and 59 to 64 days after transplanting at the coastal and inland site, respectively. To minimize the potential effect of diurnal fluctuation in PPGs (phenylpropanoid glycosides), we sampled from 9am until 1pm, and to minimize the effect of leaf position, we sampled 2 leaves from the 3rd node when possible, using leaves from the 4th and 5th nodes if leaves at the 3rd node were damaged. After sampling, leaves were flash-frozen with liquid nitrogen and then freeze-dried. For samples that did not meet the minimum dry mass (3mg), we either pooled them with other low-mass samples (by grouping within all fixed and random factors as discussed below) or excluded them. Our final sample size was 216 from Bodega (perennials only due to high annual mortality at Bodega) and 599 from Pepperwood. To determine the PPG concentrations in sampled leaves, we ground, extracted, and prepped extract aliquots as described in Holeski et al. (2013). We then used high-performance liquid chromatography (HPLC) to quantify PPGs. The HPLC method is described in (Kooyers et al., 2017) and was run on an Agilent 1260 HPLC with a diode array detector and Poroshell 120 EC-C18 analytical column [4.6□×□250□mm, 2.7 µm particle size]; Agilent Technologies). We calculated concentrations of individual PPGs as verbascoside equivalents, using a standard verbascoside solution (Santa Cruz Biotechnology, Dallas, Texas), as described in (Holeski et al., 2013, 2014).

Statistical analyses

We performed all statistical analysis in R version 4.3.1 (R core team 2023). We addressed the following main questions within each transplant site: Do annuals and perennials differ in allocation to reproduction, allocation to herbivore resistance, and fitness? Do hormones and herbivores influence allocation to reproduction, allocation to herbivore resistance, and local adaptation?

Measurements of allocation & adaptation

To compare allocation to reproduction, we measured the onset of flowering for each plant. Earlier-flowering plants invest in reproductive tissues at a time when other plants are allocating all energy to growth and defense. To compare allocation to herbivore resistance, we measured the presence or absence of herbivore-attack for each plant and the concentration and composition of the defensive compounds PPGs in leaves. Finally, to determine adaptation in each environment, we measured survival across the season, the presence of flowers, and seasonal fruit production. For annuals, these measures indicate lifetime fitness, whereas perennials that survived the season have the potential to reproduce in subsequent years.

Univariate analysis

Within each transplant site, we fit mixed effect models for each analysis that included ecotype, hormone treatment, and exclosure type as interactive fixed factors. The response variables were flowering time, the presence or absence of herbivory, survival, total PPG concentration (summed concentration for all PPG compounds), whether an individual produced a reproductive structure (buds, flowers, or fruit), or the number of fruits produced by plants that flowered at the end of the season. All models also included two random effects for maternal family and experimental plot. We fit all mixed models except for the survival model with the R package *glmmTMB* (Brooks et al., 2017), and modeled survival using the R package *coxme* (Therneau, 2022). We identified the best fitting error distributions by evaluating model diagnostics with the R package *DHARMa* (Hartig, 2022). We fit mixed models for flowering time with gaussian error distributions, mixed models for herbivory and flowering probability with binomial error distributions, and mixed models for log-transformed total PPGs with gamma distributions. We modeled survival using a mixed effect Cox Proportional Hazards model, and modeled fruit number with a zero-inflated negative binomial error distribution at the coastal site and a negative binomial error distribution at the inland site. To prevent model overfitting, we used an analysis of deviance (Wald χ^2 test) to assess the significance of model terms and sequentially removed unsupported model terms (R package *car*, *(Fox & Weisberg, 2018)*. We compared fits of complex versus reduced models using likelihood ratio tests (LRT) to find the minimum adequate model for each response variable in each site (Tables S1 and S2). We compared treatment groups using post-hoc tests on the minimum adequate model with the R package *emmeans* (Lenth et al., 2020). No contrasts were performed on predictor variables that were not in the minimum adequate model. We predicted the mean and 95% confidence intervals for each response variable from our models using the R package *ggeffects* (Lüdecke, 2018). For all non-binary response variables, we predicted confidence intervals via bootstrapping (*n*=500 iterations). We plotted raw data and predictions in *ggplot2* (Wickham, 2016) and combined plots with *patchwork* in R (Pedersen, 2019).

Multivariate analysis

Within each transplant site, we modeled the concentration of all nine different PPGs (the PPG arsenal) using mixed effect models for each analysis that included ecotype (at Pepperwood only), hormone treatment, and exclosure type as interactive fixed factors and block as a random factor. We fit all models with PERMANOVA with Bray-Curtis distance using the adonis2 function from the R package *vegan* (Oksanen, 2016). We dropped all non-significant factors for the minimum adequate model (Table S3). To test for homogeneity of variance among treatment groups, which can influence inference, we used the betadisper function from the *vegan* package. The only factor that had heterogeneity of variance among levels was ecotype. Due to the strength of the signal for ecotype, and confirmation from other studies that annuals and perennials have different PPG arsenals (Holeski et al., 2013), we are confident that differences due to ecotype are not attributable only to heterogeneity of variance. We compared treatment groups using post-hoc tests on the minimum adequate model with the function pairwise.adonis2 from the R package *pairwiseAdonis* (Arbizu, 2019). To visualize how multivariate PPG composition is influenced by our factors, we used non-metric multidimensional scaling (NMDS) (*MetaMDS* function in *vegan* package with Bray-Curtis distance to determine dissimilarity) and added standard-error ellipses at 95% confidence around the centroid of each cluster (function *ordiellipse* from package vegan).

Results

Do annuals and perennials differ in allocation to reproduction and vegetative growth (through differences in the onset of flowering)? Do hormone treatments or herbivore exclosures affect allocation?

At both sites, annuals had greater allocation to reproduction, flowering significantly earlier than perennials. Hormones did influence this allocation slightly, though only for annuals; annuals treated with GA (at both sites) and paclobutrazol (at the coast only) showed delayed flowering relative to controls. Herbivory (in control structures vs exclosures) did not influence allocation to reproduction.

At the coastal site, gibberellic acid (GA) and paclobutrazol slightly, but significantly, delayed annual flowering time relative to control annuals (plants sprayed with 0.25% ethanol). Paclobutrazol-treated and GA-treated annuals flowered 10-17 days later than controls (Fig. 2A, Tukey post-hoc tests: Table S3). However these effects were small relative to ecotype differences in flowering time: all annuals flowered 46 to 64 days earlier than their corresponding hormone treated perennials, all significant differences (Table S4).

At the inland site, in the control structures only, GA slightly, but significantly, delayed annual flowering time relative to the control. GA-treated annuals in control structures flowered 9 days later than control annuals in control structures (Fig. 2B, Tukey post-hoc tests: Table S4). Again, this effect was small relative to ecotype differences in flowering time: all annuals flowered 39 to 62 days earlier than their corresponding hormone-treated perennials in both the exclosures and control structures, all significant differences (Table S5).

Hormone treatments had no effect on perennial flowering time relative to controls at either transplant site (Tables S4 & S5). Exclosures had no effect on flowering time at either transplant site (Tables S1, S2, S4, S5).

Do annuals and perennials differ in allocation to herbivore resistance (via changes in the probability of herbivore attack)? Do hormone treatments or herbivore exclosures affect allocation?

At both sites, and contrary to expectations, perennials were more likely to experience herbivory than annuals (excluding the exclosures at the coast, in which both ecotypes experienced equivalent chances of herbivory). GA was the only hormone to influence the probability of herbivory, and only at the coast, where, again contrary to expectations, it reduced the probability of herbivory for both ecotypes. This is likely due to an interaction with salt-spray resistance rather than allocation to herbivore resistance.

Perennials were significantly more likely to be damaged by herbivores than annuals in the control structures at the inland site, and in both control structures and exclosures at the coastal site, but the difference between ecotypes was smaller in the exclosures (Table S6 and S7). At the inland site, herbivores damaged 42% (126/300) of perennials and 13% (38/300) of annuals in the control structures and 33% (65/200) of perennials and 13% (26/200) of annuals in the exclosures. At the coastal site, herbivores damaged 74% (221/300) of perennials and 4% (12/300) of annuals in the control structures and 48% (96/200) of perennials and 9% of annuals (17/200) in exclosures. However, these numbers are somewhat misleading at the coastal site, since annuals perished quickly due to salt spray and had less time to encounter herbivores and accrue herbivory.

The mesh-size of screen used in our exclosures, while necessary to allow salt spray to enter, did allow some small insects, including leaf miners and weevils, to enter the exclosures (or they were present when the exclosures were erected) and damage plants mildly. As a result, our herbivore exclosures did not significantly reduce the probability of insect herbivory for perennials at either transplant site, although they were highly effective at reducing herbivory from deer and voles that removed flowering stalks from plants, greatly impacting fecundity.

At the coastal site, GA-treatment reduced the probability of herbivore attack in both annuals and perennials, likely due to GA effects on survival and salt spray sensitivity detailed below. The only effect of exclosure was increasing herbivory probability for GA-treated annuals, though again this is likely due to an interaction with salt-spray (Figure 2C, Tukey post-hoc tests: Table S6). At the inland site, hormone-treated annuals and perennials did not significantly differ from their respective controls and exclosures did not influence the probability of herbivory for either ecotype (Tukey post-hoc tests: Table S7).

Do annuals and perennials differ in allocation to herbivore resistance (via changes in PPGs)? Do hormone treatments or herbivore exclosures affect allocation?

Perennials showed greater allocation to herbivore resistance (via PPG concentration) than annuals at the inland site, while annual mortality at the coast prevented this comparison. Herbivory (in control structures vs exclosures) had limited impacts on PPGs, moderating the effects of hormone treatments at the coast and influencing multivariate PPG arsenals inland. GA influenced PPG allocation at both sites (negatively at the coast and positively inland) and methyl jasmonate increased PPG allocation inland.

At the inland site, perennials had significantly higher total PPG concentration than annuals (Tukey post-hoc tests: Table S9) and annuals and perennials differed in their multivariate PPG arsenals. The effect of ecotype was generally stronger than any hormone effects. We were unable to compare annuals and perennials at the coastal site due to high annual mortality.

At both sites, exclosures had no effect on total PPG concentration (Table S1), though exclosures did moderate the effect of hormone treatment at the coastal site (Table S2). Exclosure did not influence the multivariate PPG arsenal at the coastal site but did at the inland site (Table S3). At the coast, the only effect of hormone treatment was that GA reduced total PPG concentration of perennials in the control plots (Figure 3a, Tukey post-hoc tests, Table S8) and caused the PPG arsenal to differ from control plants (Figure 3c, PERMANOVA pairwise, Table S10). While this impact of GA is consistent with our predictions that GA downregulates defense-allocation, it is also possible that the decrease in total PPG is due to increased salt-stress experienced by GA-treated plants. Inland, hormone treatments did not influence PPGs in perennials (Figure 3b,c, Table S9). In annuals at the inland site, GA and MeJa increased the total concentration of PPGs (Figure 3b, Table S10) and caused the PPG arsenal to differ (Figure 3d, PERMANOVA pairwise, Table S11). While we expected MeJa to increase allocation to defense, we expected GA to decrease it. However, the increase in total PPG is consistent with an increase in days to flowering in GA-treated annuals at the inland site (these traits positively covary in annuals, (Kooyers et al., 2020), though the mechanism for this shift is unknown.

Do annuals and perennials differ in fitness components (survival, the probability of flowering, and fruit number)? Do hormone treatments or herbivore exclosures affect fitness components and homesite advantage?

Perennials survived significantly longer than annuals at both sites. At the coast, while annuals and perennials were equally likely to flower, the vast majority of annuals were killed by salt before producing fruits. Inland, annuals were more likely to flower and produce fruits than perennials, though perennials that did flower produced as many fruits as annuals. Herbivory (in control structures vs exclosures) influenced the probability of flowering and fruit production only at the coast where herbivory resulted in reproductive failure of perennials outside of exclosures. In general, GA had a negative effect on fitness at both sites, though impacts varied by fitness component and ecotype across sites. At the coast, GA reduced survival relative to controls by increasing susceptibility to salt spray and reduced the probability of flowering for both ecotypes. Inland, GA and MeJa reduced the probability of flowering for perennials and GA reduced fruit production in both ecotypes.

GA-treatment reduced survival due to oceanic salt spray at the coastal site

At both transplant sites, perennials had significantly higher survival by the end of the experiment than annuals (coastal site: 1% (4/500) of annuals and 77% (383/500) of perennials survived; inland site: 2% (10/500) of annuals and 21% (106/500) of perennials survived, Tukey post-hoc tests: Table S12 and S13). At the coastal site, salt spray was the only source of mortality for annuals and 93% (109/117) of the perennials that died. For the remaining 8 perennial plants, the source of mortality was attributed to herbivory. The main source of mortality at the inland site was the onset of summer drought.

At both transplant sites, exclosures had no effect on survival (Figure 4, Tables S1, S2 and S12). Hormone treatments had no effect on survival at the inland site (Table S1), but GA treatment significantly reduced survival for both ecotypes relative to their respective controls at the coastal site (Figure 4, Table S12). GA treatment reduced survival for both ecotypes at the coastal site by increasing susceptibility to salt spray. GA-treated perennials were also more upright compared to prostrate controls, and elongated their stems early in development like annuals which may have increased exposure to salt spray (Figure 5).

GA-treatment reduced flowering probability at the coastal site

Despite high mortality due to salt spray at the coastal site, annuals and perennials did not significantly differ in the probability of flowering (Tukey post-hoc tests: Table S14). Due to their rapid phenology, 40% (198/500) of annual transplants were able to flower prior to dying of salt spray, although death occurred quickly after flowering so very few annuals produced fruit (detailed below). Herbivore exclosures significantly increased the probability of flowering for perennials (control structures: 27% (80/300) of perennials and 26% (77/300) of annuals flowered; exclosures: 74% (148/200) of perennials and 61% (121/200) of annuals flowered, Table S14), which may be due to the reduction of large mammalian herbivory and/or minor buffering of salt spray from condensation collecting on mesh screens. In addition, GA treatment reduced the probability of flowering for both ecotypes relative to their respective controls, likely due to the effect of GA on sensitivity to salt spray (Figure 6A, Table S14).

At the inland site, annuals had a significantly higher probability of flowering than perennials, and exclosures had no effect on the probability of flowering for either ecotype (control structures: 39% (117/300) of perennials and 92% (277/300) of annuals flowered; exclosures: 66% (133/200) of perennials and 100% (200/200) of annuals flowered, Tukey post-hoc tests: Table S15). GA and MeJa treatment reduced the probability of flowering in perennials, but hormone treatment did not affect the probability of flowering in annuals (Figure 6B, Table S15).

Herbivore exclosures drastically increased fruit production at the coastal site

At the coastal site, only plants protected by exclosures successfully produced fruit by the end of the season (annuals: 0% (0/300) produced fruit in control structures and 4% (7/200) produced fruits in the herbivore exclosures; perennials: 0% (0/300) produced fruit in control structures and 60% (119/200) produced fruits in the herbivore exclosures). The reason that none of the plants outside of the exclosures produced fruits was because of complete herbivory of the inflorescences of these plants by mule deer (*Odocoileus hemionus*). Since no plants produced fruit outside of the exclosures, and few annuals produced fruit inside the exclosures (annuals in exclosures: *n*=3 controls, *n*=1 GA-treated, *n*=3 paclobutrazol-treated), we analyzed only the effect of hormone treatments on perennial fruit production inside the exclosures at the coastal site (Figure 6C). Fruit production in perennial plants that flowered in exclosures at the coastal site was not significantly associated with hormone treatment (Table S1).

At the inland site, 88% (265/300) of annuals and 29% (86/300) of perennials produced fruit in the control structures, while 100% (200/200) of annuals and 53% (105/200) of perennials produced fruit in the exclosures. The majority of plants that flowered produced fruit: 96% (265/277) of annuals and 74% (86/117) of perennials that flowered produced fruit in the control structures, while 100% (200/200) of annuals and 79% (105/133) of perennials that flowered produced fruit in the exclosures. However, annuals and perennials that flowered did not significantly differ in fruit production in either control structures (mean fruit number for flowering annuals: 4.6, flowering perennials: 5.7) or exclosures (mean fruit number for flowering annuals: 7.6, flowering perennials: 7.8; Tukey post-hoc tests: Table S16). GA treatment significantly reduced fruit production in both ecotypes that flowered relative to their respective controls (difference between control and GA-treatment for perennials: 5.8 fruit in exclosures, 2.3 in controls; and for annuals: 3.3 fruit in exclosures, 0.7 in controls; Figure 6; Table S16).

Discussion

Across environmental gradients, shifts in allocation between reproduction, growth, and defense have been found to follow predictable patterns, suggesting that these shifts underlie local adaptation (Bazzaz et al., 1987; Hahn & Maron, 2016; Züst & Agrawal, 2017). However, multiple abiotic and biotic factors co-vary across environmental gradients and multiple traits often differ between locally adapted populations, making the identification of selective agents and their phenotypic targets a major challenge (Wadgymar et al., 2017, 2022). In this study, we used a manipulative reciprocal transplant experiment to test the hypothesis that herbivory and divergence in allocation to reproductive timing, vegetative growth, and defense against herbivores contributes to local adaptation across a coastal to inland environmental gradient. Growing seasons are shorter in inland environments, which generates selection for earlier reproduction. At our coastal site, herbivore exclosures dramatically increased fecundity of local coastal perennials, but contrary to our predictions, did not contribute to local adaptation. This is likely due to the abiotic effect of salt-spray pre-empting the impacts of herbivory on annuals. Our hormone treatments slightly shifted allocation between vegetative growth, reproduction and defense in each ecotype, but did not recapitulate the full effect size of differences previously observed in controlled greenhouse conditions (Lowry et al., 2019). Nevertheless we observed dramatic effects of our hormone treatments on survival and fecundity across our transplant sites. Despite delaying flowering, the GA application caused obviously earlier bolting and taller heights in the perennial transplants. This earlier bolting, and possibly other physiological changes, may have been responsible for the increased mortality due to salt spray on the coast in both ecotypes, and salt spray was the primary (>99%) source of mortality for transplants at our coastal site. Our results suggest that divergence in salt spray tolerance, potentially mediated by ecotype differences in gibberellin synthesis/sensitivity, is an important driver of local adaptation to coastal habitats.

Role of biotic interactions in local adaptation

The organisms a plant interacts with vary across the landscape, causing different selective pressures (Friberg et al., 2019; Thompson, 2005; Urban, 2011). Given the differences in the abiotic environment at our two sites - cool and foggy on the coast, hot and dry inland - the communities of organisms which our plants interact with differ substantially. The moist coastal environment has far more molluscan herbivores (snails, slugs), and a rare leaf-mining fly (Eiseman et al., 2023), which we did not see at the much drier Pepperwood Preserve. Voles and deer also contribute to herbivory at the coastal site only. Given the differences in communities, differences in defense-levels, and prior research suggesting differences in intensity of herbivory on the coast and inland (Holeski, 2007), we predicted herbivory would be a driving factor in local adaptation. Remarkably, we found no effects of herbivory on local adaptation at these two specific sites; nevertheless, we stress that insect-plant interactions regularly occur in a complex mosaic across the landscape and vary temporally (Rotter et al., 2022).

High rate of herbivory at the coastal site did not contribute to local adaptation

At the coast, we predicted that high rates of herbivore attack would result in herbivory playing a strong role in local adaptation. Although we observed high rates of herbivory, reducing herbivory with exclosures did not increase local adaptation because of the effect of an abiotic factor, oceanic salt spray. Annuals transplanted on the coast quickly exhibited necrosis from salt spray before dying; the window that they could have received herbivory was short, and they were likely poor quality host plants during that time. The perennials, in comparison, were larger, healthier, and had many more days in which to encounter an herbivore and receive damage. Ephemeral plants are more likely to escape herbivory (Feeny, 1976), and all reproductive herbivory at the coastal site came after the median death date of our annual plants. This pattern highlights the importance of the timing of selective events, particularly for local adaptation of ecotypes with differing life-history strategies. The importance of fecundity versus survival are likely to differ between ecotypes (DeMarche et al., 2016), and early-season factors (like coastal salt spray) that impact survival might disproportionately contribute to fitness differences between populations relative to a late season factors that influence fecundity (such as herbivory) (Crone, 2001; Wadgymar et al., 2022).

While this study suggests that herbivory is preempted from playing a role in keeping annuals out of coastal environments, it does not mean it is unimportant. In the control structures on the coast, perennials completely failed to reproduce due to deer herbivory of inflorescences. By virtue of allocating growth to clonal expansion and non-reproductive tissue, perennials are likely increasing both tolerance (Stevens et al., 2008) and temporally escaping herbivory. Some populations are completely sterilized (i.e., all inflorescences are completely consumed by herbivores) in certain years (Toll, pers. obs.), and thus herbivory may be an extremely strong selective pressure in the morphology, allocation to clonal growth, and reproductive timing of these coastal perennials. The results of this study were also clearly influenced by the close proximity of our coastal field site to the open ocean (within 50 meters of the shoreline) While it is common for coastal perennials to grow in close proximity to the ocean, where they are impacted by high levels of salt spray, it is also common for them to grow slightly further inland, where salt spray is greatly reduced (Barbour, 1978; Boyce, 1954; Du & Hesp, 2020).

Life history contributed to differences in herbivore attack at the inland site

Our finding that herbivory did not influence local adaptation inland is somewhat less surprising, as there is evidence that herbivore damage is generally less extensive there (Holeski, 2007). It was unexpected, however, that perennials were also more likely to be attacked by herbivores than annuals at the inland site, as we predicted that perennials would be more resistant to herbivory due to ecotype differences in phytochemical defenses (PPGs). At our inland site, however, perennials were more likely to be attacked even during periods of time when both ecotypes were alive in the same site. The general (though non-significant) trend for the homesite advantage of annuals to decrease in the exclosures relative to the control structures, may suggest that herbivory, in some years, does contribute to local adaptation inland. Higher attack rates for perennials could be due to differences in apparency caused by differences in plant size (Feeny, 1976), or herbivore preference due to nutritional differences or water content. In addition, while PPGs are feeding deterrents to generalists, some can be feeding stimulants for specialist herbivores (Holeski et al., 2013; Rotter et al., 2018), and thus perennials may be more likely to get attacked by specialists. Our presence-absence measure of herbivory also may have missed differences in degree of herbivory among plants that were attacked, which may have greater impacts on fitness.

Hormone pathways underlying local adaptation

Oceanic salt spray sensitivity increased with gibberellin treatment

The most surprising result of our experiment was how dramatically GA3 application decreased survival of coastal perennial genotypes at the coastal field site. Based on the patterns of damage, necrosis of plant tissue, we attributed this mortality primarily to oceanic salt spray. There are two non-mutually exclusive ways that GA3 could have decreased fitness in the coastal environment with regard to salt spray. First, the addition of GA3 increased plant height, as evident by increased internode elongation of plants (Lowry et al., 2019). Increased plant height could put the aboveground portions of these plants more directly in the path of prevailing wind delivering the salt spray (Zambiasi & Lowry, 2023). A second hypothesis is that the GA3 treatment may directly increase susceptibility of tissues to salt spray independent of changes in plant height. The second hypothesis is particularly intriguing, as it is the opposite of what would be expected based on the soil salinity literature. For example, previous experiments in rice (Rodríguez et al., 2006), wheat (Iqbal & Ashraf, 2013), apple (X. Wang et al., 2019), cucumber (Y. Wang et al., 2020), and sorghum (J. Liu et al., 2023) have all found that the application of GA3 increases yields under saline conditions. The conflicting results of those studies and our experiment make it clear that findings from the soil salinity literature cannot be directly extrapolated to what is experienced by plants growing in coastal environments, where salt spray is a major source of stress on plant aboveground tissues (Boyce, 1954; Du & Hesp, 2020; Itoh et al., 2024). The exact mechanisms by which GA3 increases salt spray susceptibility are still not clear, but are an active focus of our current research. One possibility is that the addition of GA3 increases stomatal size and/or opening (X. Liu & Hou, 2018; Nir et al., 2017; Shohat, Cheriker, et al., 2021; Shohat, Eliaz, et al., 2021), which allows for more salt spray to enter leaves.

Oceanic salt spray preempted herbivory

Our hormone treatments altered the probability of herbivore attack at the coastal site, however, this was not likely due to an increase in allocation to resistance. GA-treated perennials were less likely to be attacked by herbivores (Figure 2), but were also less salt spray tolerant (Figure 4) than control perennials at the coastal site. GA treatment was not associated with Total PPG concentrations in the exclosures at the time of tissue sampling, but GA-treated perennials had lower Total PPG concentrations than control perennials in the control structures at the coastal site (Figure 3). Thus, the observed decrease in herbivory was likely due to a decrease in tissue quality induced by salt spray stress. GA-treated plants also senesced and died faster in the control structures at the coastal site; the median death date in the control structures was 53 days compared to 96 days in the exclosures (Figure 4). Coastal fog sometimes condensed on the screens that we used to exclude herbivores, which may have slightly decreased the transmission of salt spray into exclosures. Eventually, individuals in both the exclosures and controls showed evidence of salt spray damage and death, but the lag in the onset of damage may also partially explain why we observed a reduction in total PPGs in the control structures but not the exclosures at the time of sampling.

Hormone effects were attenuated in the field

Aside from salt spray tolerance, the effects of our hormone applications on measured phenotypes were markedly weaker than we expected from greenhouse experiments. The limited impact on flowering time and the notable impact on growth habit are consistent with a previous greenhouse study (Lowry et al., 2019). In that same greenhouse study, however, daily spraying of GA on perennial monkeyflowers reduced the concentration of PPGs. In our study, GA only reduced the concentration of PPGs in the control structures at the coast (Figure 3), though it did alter the PPG arsenal of perennials at both sites, albeit not dramatically. We also expected a greater impact of MeJa, an antagonist of GA that induces plant defense (Baldwin, 1998; Kessler & Baldwin, 2002). This may be due in part to methodological constraints imposed by field studies. The difficulty of preventing cross-contamination of nearby plants prevented us from repeatedly treating our transplants with hormones after field planting, which may have weakened and/or attenuated the effects compared with long-term applications (Hummel et al., 2009). Also, interactions with environmental conditions in the field (e.g., short days, cold nights, and greater temperature variation relative to greenhouse conditions) may have impacted our measured traits more than the hormone treatments. For example, temperature interacts strongly with GA-pathways to control phenology and development (Penfield, 2008) and PPG production in monkeyflowers is influenced by temperature and day-length (Blanchard et al. in review). Thus, while our finding that GA impacts local adaptation via salt-tolerance supports the value of field-based hormone treatments, our study also suggests the need for field-based preliminary trials to determine field-relevant doses.

Conclusions

Interactions among hormone pathways mediate differences in allocation to growth, reproduction, and resistance, but few studies have investigated how evolutionary changes in hormone pathways contribute to local adaptation (James et al., 2023; Wilkinson et al., 2021). Evidence that GA application reduced allocation to resistance in greenhouse experiments led us to hypothesize that selection by herbivores drove the evolution of GA-suppression in coastal perennials. Unexpectedly, we found that GA application reduced local adaptation of perennials at the coast by making them more susceptible to salt spray and that coastal salt spray killed all annuals. This suggests a strong role for an abiotic factor, salt spray, in selecting for differences in GA pathway genes in coastal populations. Additionally, herbivory had a dramatic impact on perennial fecundity at the coast, though it was precluded from contributing to local adaptation by the salt spray induced mortality of all annuals at the coast. While our study shows how hormone applications can be used to investigate the mechanisms underlying local adaptation, our results also stress the importance of considering the interaction and timing of selective agents.

Figure Legends

Figure 1. Photographs depicting transplant sites and structures used in the reciprocal transplant experiment. For our reciprocal transplant experiment, each site had ten plots, each with 100 plants. Each site had six control plots (with mesh tops and partially-mesh sides) and four exclosure plots (fully enclosed with mesh on all sides). A control and exclosure plot are shown side by side in the foreground of the inland site.

Figure 2. Allocation to reproduction and defense: flowering time and probability of herbivory of annuals (circles) and perennials (triangles) treated with gibberellic acid (GA, yellow), paclobutrazol (Paclo, blue), and methyl jasmonate (MeJa, green), and the controls (0.25% ethanol, black) in control structures and herbivore exclosures at the coastal site, Bodega Marine Reserve (A & C), and the inland site, Pepperwood Preserve (B & D). Larger symbols in the foreground are the mean predictions and 95% confidence intervals from the minimum adequate mixed effect models, smaller and lighter symbols in the background are the raw data. Results of Tukey post-hoc contrasts within each site are indicated above each prediction; shared letters indicate that groups do not significantly differ, while non-overlapping letters indicate that groups significantly differ within each site. Exclosure type was not plotted for flowering time on the coast (A) because the minimum adequate model did not include exclosure type as a fixed effect.

Figure 3. Allocation to chemical defense: total concentration of all PPGs and differences in multivariate PPG arsenals of annuals (circles) and perennials (triangles) treated with gibberellic acid (GA, yellow), paclobutrazol (Paclo, blue), and methyl jasmonate (MeJa, green), and the controls (0.25% ethanol, black) in control structures and herbivore exclosures at the coastal site, Bodega Marine Reserve (A & C), and the inland site, Pepperwood Preserve (B & D). In the Total PPG figures (A & B), larger symbols in the foreground are the mean predictions and 95% confidence intervals from the minimum adequate mixed effect models, smaller and lighter symbols in the background are the raw data. Exclosure type was not plotted for Total PPG at the inland site (B) because the minimum adequate model did not include exclosure type as a fixed effect. PPG arsenal figures (C & D) use non-metric multidimensional scaling (NMDS) with Bray-curtis distance (and 95% confidence interval ellipses) to visualize multivariate differences among plants. Exclosure type was either not a significant factor in the multivariate model (C) or did not interact with other fixed effects (D) and was therefore not included in these plots.

Figure 4. GA application decreased survival at the coastal site. Survival probabilities for annual (solid line) and perennial (dashed line) transplants at the coastal site, Bodega Marine Reserve (A), and the inland site, Pepperwood Preserve (B). Survival probabilities and 95% confidence intervals for control (black), GA (yellow), methyl jasmonate (blue) and paclobutrazol (green) treatments were predicted from Cox Proportional Hazards models. At the inland transplant site (B), survival probabilities and 95% confidence intervals were only plotted for ecotypes (grey)

because the minimum adequate model did not include exclosure or hormone treatment as a fixed effect. Results of Tukey post-hoc contrasts within each site are indicated above each final predicted survival; shared letters indicate that groups do not significantly differ, while non-overlapping letters indicate that groups significantly differ within each site.

Figure 5. GA application on perennials resulted in stem-elongation relative to controls. The plants pictured (on day 16 after transplantation) are from the same family and were grown in the same plot at the coastal site.

Figure 6. Herbivore exclosures tended to increase, while GA applications tended to decrease components of fecundity: probability of flowering and fruit number of annuals and perennials that flowered treated with gibberellic acid (GA, yellow squares), paclobutrazol (Paclo, blue diamond), and methyl jasmonate (MeJa, green triangle), and the controls (0.25% ethanol, black circles) in control structures and herbivore exclosures at the coastal site, Bodega Marine Reserve (A & C), and the inland site, Pepperwood Preserve (B & D). Larger symbols in the foreground are the mean predictions and 95% confidence intervals from mixed effect models, smaller and lighter symbols in the background are the raw data. Results of Tukey post-hoc contrasts within each site are indicated above each prediction; shared letters indicate that groups do not significantly differ, while non-overlapping letters indicate that groups significantly differ within each site. Predictions are not plotted for fruit production at Bodega Marine reserve because no fixed factors were in the minimum adequate model. To improve visualization, one outlier that produced 164 fruit was not plotted at the Bodega Marine Reserve.

Figure 1.

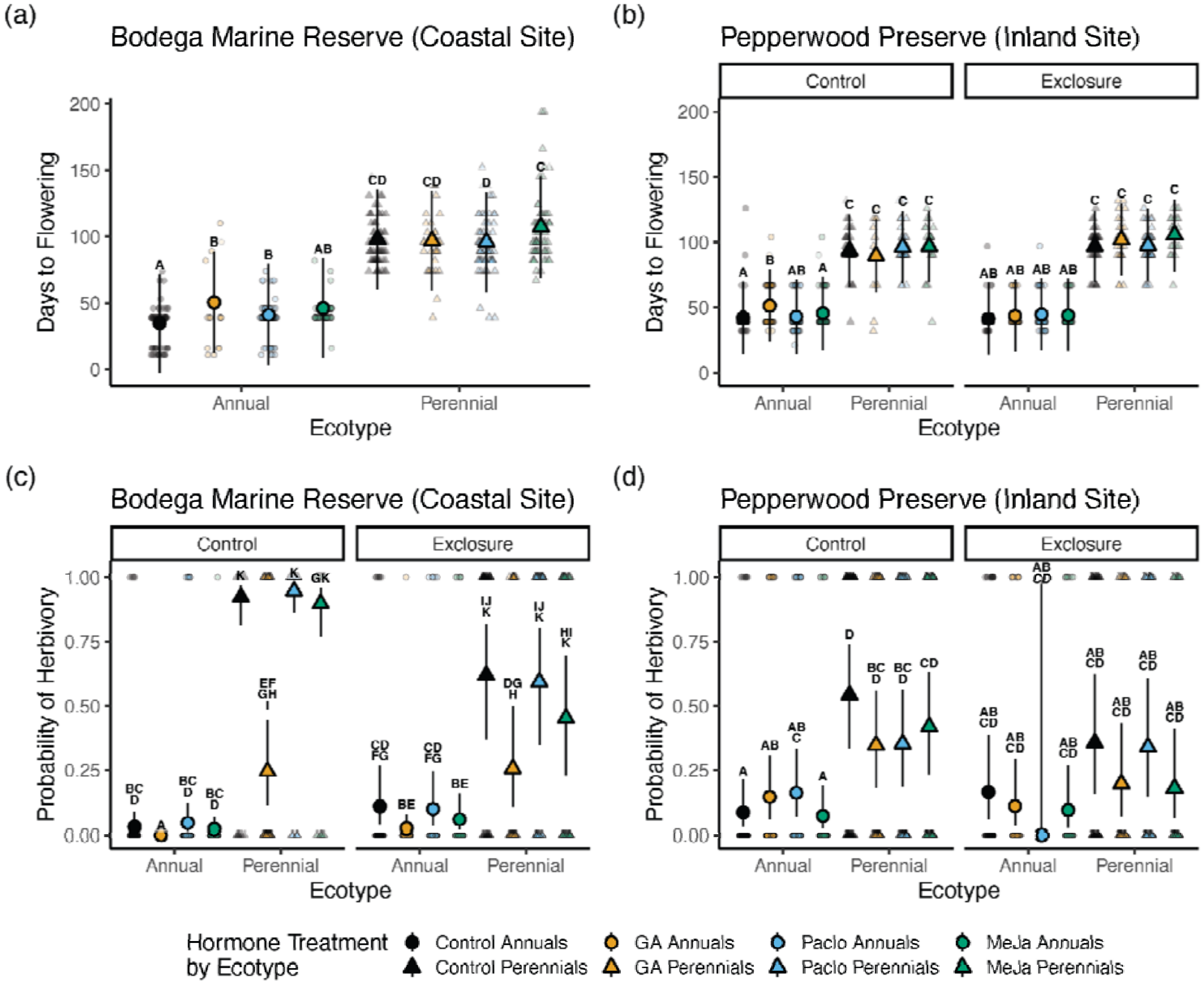
(a)
Bodega Marine Reserve (Coastal Site)
Days to Flowering
0
50
100
150
200
A
B
B
AB
CD
CD
D
C
Annual
Perennial
Ecotype
(b)
Pepperwood Preserve (Inland Site)
Control
Exclosure
Days to Flowering
A
B
AB
A
C
C
C
C
AB
AB
AB
AB
C
C
C
C
Annual
Perennial
Annual
Perennial
Ecotype
(c)
Bodega Marine Reserve (Coastal Site)
Control
Exclosure
Probability of Herbivory
0.00
0.25
0.50
0.75
1.00
BC D
A
BC D
BC D
K
EF GH I
K
GK
CD FG
BE
CD FG
BE
IJ K
DG H
IJ K
HI K
Annual
Perennial
Annual
Perennial
Ecotype
(d)
Pepperwood Preserve (Inland Site)
Control
Exclosure
Probability of Herbivory
A
AB
AB C
A
D
BC D
BC D
CD
AB CD
AB CD
AB CD
AB CD
AB CD
AB CD
AB CD
AB CD
Annual
Perennial
Annual
Perennial
Ecotype
Hormone Treatment by Ecotype
Control Annuals
Control Perennials
GA Annuals
GA Perennials
Paclo Annuals
Paclo Perennials
MeJa Annuals
MeJa Perennials

Figure 2.

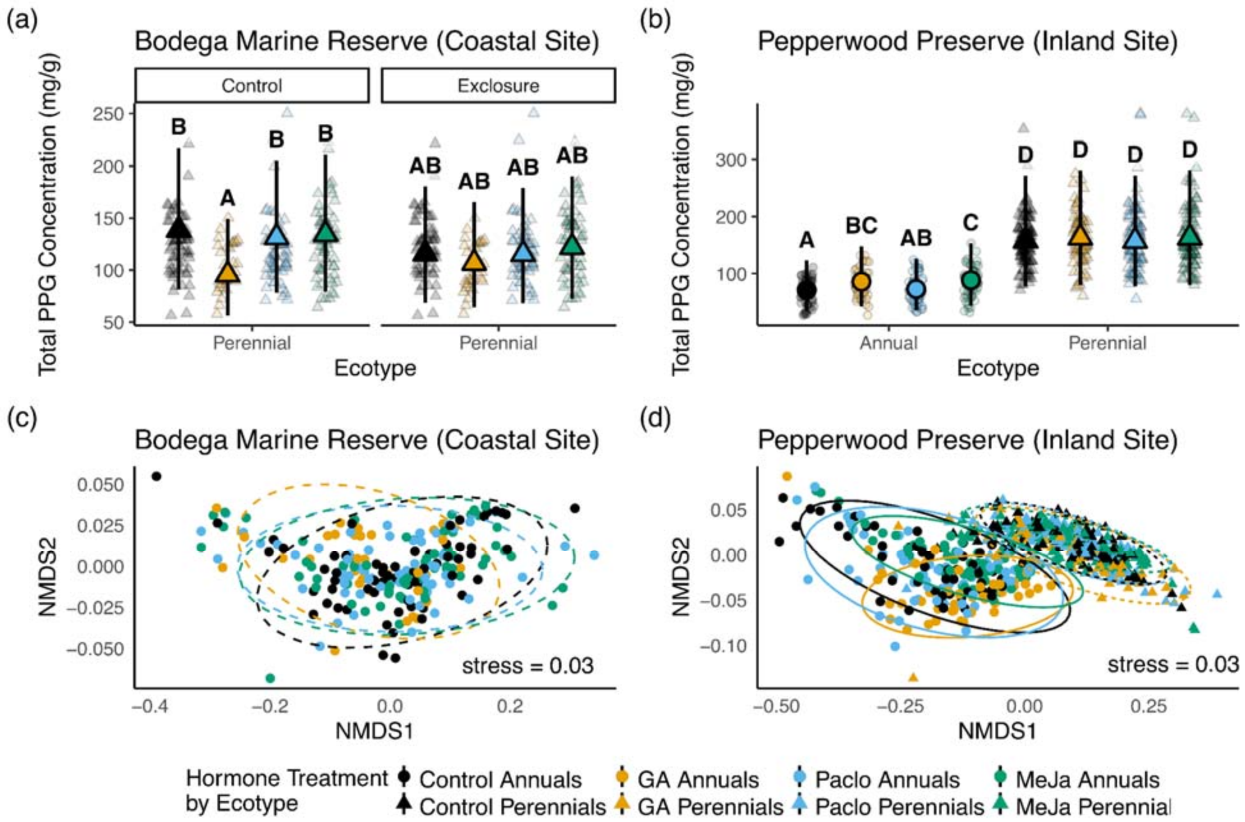

Figure 3.

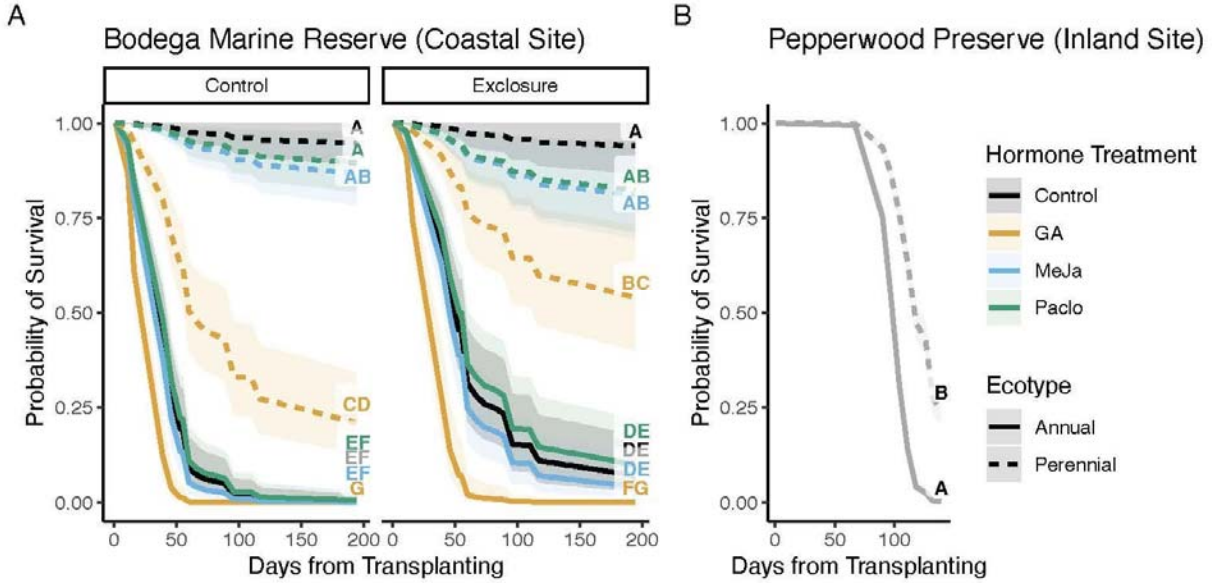

Figure 4.

Figure 5.

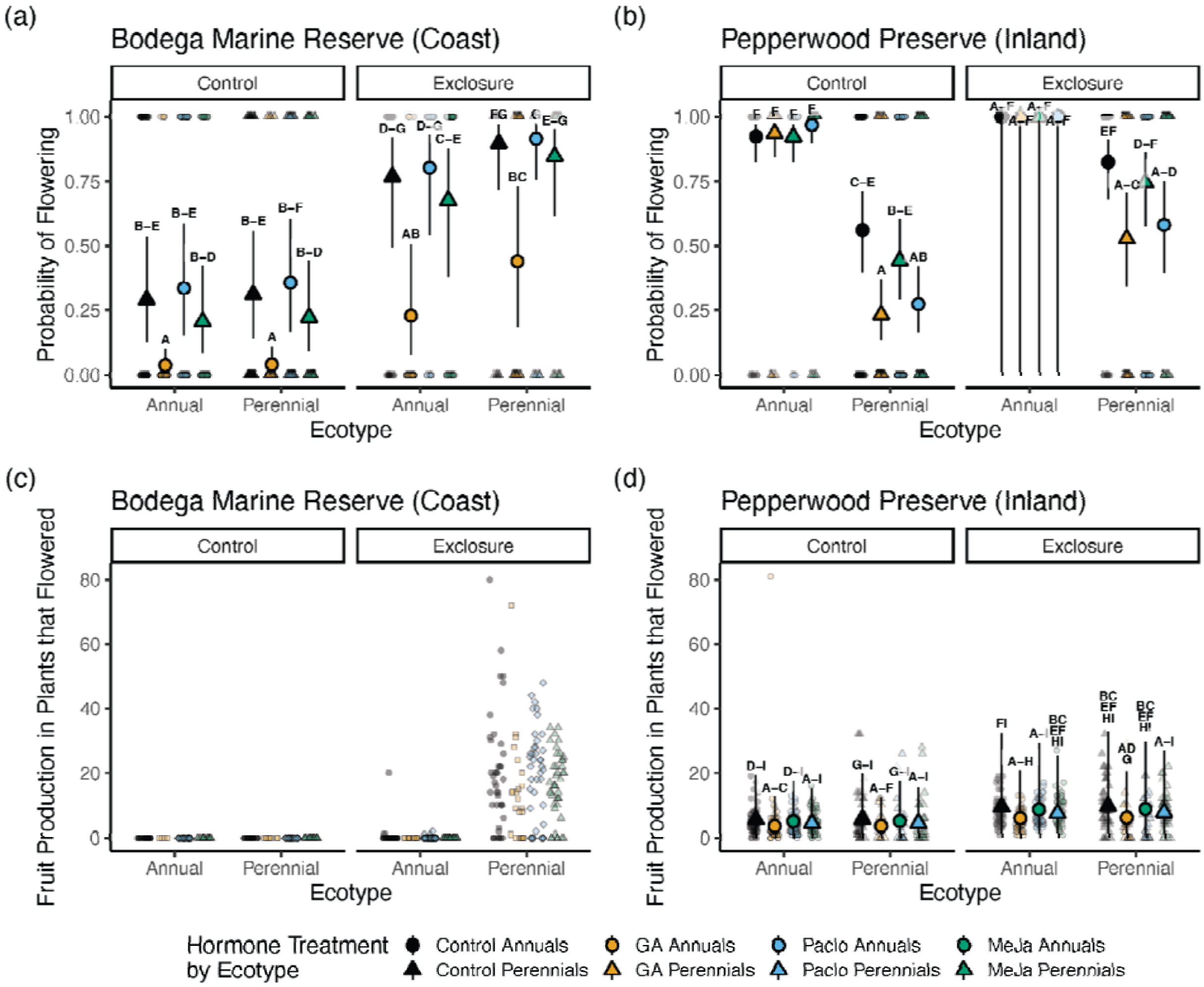
(a)
Bodega Marine Reserve (Coast)
(b)
Pepperwood Preserve (Inland)
Control
Exclosure
Probability of Flowering
0.00
0.25
0.50
0.75
1.00
Annual
Perennial
Ecotype
(c)
Bodega Marine Reserve (Coast)
(d)
Pepperwood Preserve (Inland)
Fruit Production in Plants that Flowered
0
20
40
60
80
Hormone Treatment by Ecotype
Control Annuals
Control Perennials
GA Annuals
GA Perennials
Paclo Annuals
Paclo Perennials
MeJa Annuals
MeJa Perennials

Figure 6.

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