sections to finish, things to check in the lab, supplemental figures to add to plantago\_suppmaterials

## Introduction

Ecosystems around the world are facing rapid and unpredictable climatic changes (IPCC 2021). Over the past 50 years, many regions have faced decreased precipitation, increased temperatures, and longer durations between precipitation events (Spinoni et al. 2019). Increased number and a higher severity of droughts is expected across many landscapes in the coming decade (Dai 2011). These drought events, driven by climate change, pose a significant problem for plants populations and communities. In response to these changes, most organisms are expected to (1) migrate, (2) undergo adaptive genetic changes, and/or (3) exhibit phenotypic plasticity (Hoffmann and Sgrò 2011). However, it’s likely that most plant populations will not be able to keep pace with these climatic changes on an evolutionary scale and may not migrate fast enough to cope (Song et al. 2021). Therefore, to predict and manage biodiversity on planet earth, it is critical to understand the mechanisms that generate resilience to climate change.

Phenotypic plasticity is often viewed as the most rapid-response mechanism to environmental variation, and therefore may play a key role in plant population adaption over the next several decades as the world undergoes unprecedented change. Phenotypic plasticity is determined by its genetic inheritance and it’s immediate environment, but also by the environmental conditions experienced by parent plants, coined transgenerational plasticity (Bonduriansky, 2021; J. Herman & Sultan, 2011; Uller, 2008). Over the past two decades, it has become clear that the effects of transgenerational plasticity are diverse, widespread across organisms, and complex (reviewed in Bonduriansky, 2021; Holeski et al., 2012; Mousseau & Fox, 1998; Uller, 2008; Yin et al., 2019). For example, when exposed to two generations of drought treatments, *Secale sylvestre* exhibited higher aboveground biomass and higher seed production compared to offspring of control parental plants, increasing offspring performance (Mojzes et al., 2021). However, *Lupinus angustifolius* exposed to two generations of drought experienced reduced offspring fitness induced by parental effects by way of significantly reduced seed mass and lower reproductive biomass (Matesanz et al., 2022). While the number of studies investigating transgenerational plasticity in the past two decades has risen exponentially, no clear patterns have emerged as to the adaptivity of transgenerational effects, and several published meta-analyses have reached opposite conclusions on the perceived benefits of transgenerational plasticity (Sánchez-Tójar et al., 2020; Uller et al., 2013; Yin et al., 2019).

*What’s missing – mechanisms, environmental drivers of TGP*

Transgenerational plasticity is likely to evolve when several environmental conditions are met: environments are correlated across time, there is no cost to responding to environmental cues, and there is genetic variation available in the reaction slope norm (Colicchio & Herman, 2020a; Hoyle & Ezard, 2012; Räsänen & Kruuk, 2007; Uller, 2008) However, quantification of the genetic variation is imperative to understanding parental effects on fitness and performance traits. One confounding factor that may account for obscure results in transgenerational studies may be variance in parental effects on populations collected from environmentally distinct sites, or lack of variance in plants sourced from the same genetic pool.

*How aridity might shape population response and why it matters now*

In this study, we assessed the effects of parental drought on the annual plant *Plantago patagonica*. We studied eleven populations from abiotically distinct and contrasting environments ranging across the southwestern portion of the US (Table 1). Using a fully factorial greenhouse experiment, we implemented watering treatments that were early (10-14 days after germination) and strong (50% reduction or more of spring annual precipitation) and grew four offspring treatments that reflected all combinations of parental and offspring treatments. P. patagonica is a fitting plant to test the occurrence of transgenerational effects due to its wide natural range, over extremely ecologically and abiotically distinct environments. To assess the presence and persistence of transgenerational plasticity and how it interacts with home site atmospheric drought, we measured the phenotypic response of several performance and fitness traits. We asked the following questions: 1) Is there evidence of transgenerational plasticity in response to drought in *P. patagonica*? 2) Is the initial seed source site VPD driving the transgenerational response to drought? and 3) Is the transgenerational response to drought adaptive?

## Materials and methods

*Study species, source populations, and seed collection*

*Plantago patagonica* (woolly plantain) is a small statured, self-pollinating annual forb with a broad range, native to both North and South America. *P. patagonica* characterized by a basal cluster of hairy, grass like leaves with dense spikes of white and green flowers. In North America, *P. patagonica* is a common winter and spring annual that often forms mats across the floor in late winter and spring, depending on the climate (SEINet Portal Network, 2024). Within the southwestern portion of North America, *P. patagonica* was identified as a priority restoration species by the Bureau of Land Management’s Colorado Platea Native Plant Program due to its high likelihood for establishment in large scale projects (Wood et al., 2015), high germination probability (Gremer & Venable, 2014), its ability to seed bank (Haight et al., 2019), and its potential role in promoting perennial establishment (Barak et al., 2015). Here, we used plant material from 12 different populations of *P. patagonica*, which were passed on from a previous study by Klein & Mitchell (2023) (Figure 1).

*Experimental conditions in the parental generation (F1)*

Seeds collected from the 12 ecologically distinct populations of *P. patagonica* were grown and exposed to well-watered and ambient conditions in a common garden setting at the Petchesky Conservation Center on New Mexico Land Conservancy-owned land southwest of Santa Fe, New Mexico, USA during the spring of 2021 (35.612602, −106.004360) (F1, Figure 1). The well-watered plants received a supplemental watering equivalent to approximately 200% of the 30-year average for the experimental period (May – June 2021), and ambient watered plants received 100% of the 30-year average for the experimental period. The common garden where these plants were grown has a 30-year MAP of 331 mm and ranges annually in temperature from −0.30 to 22°C, which is intermediate in precipitation and temperature compared to collection conditions for the 12 populations (Figure 1) All home environmental characteristics were obtained using Oregon State University’s PRISM data explorer for individual locations (PRISM Climate Group). For further details on the F1 common garden experiment, see Klein & Mitchell, 2023. The offspring seed (F2, Figure 1) of these parental plants were collected, cleaned, and stored for later use.

*Experimental conditions in the offspring generation (F2)*

Seeds produced from the F1 common garden were used in the greenhouse to establish the offspring generation (F2, Figure 1) during the spring of 2023 (April – August 2023). Population 2 was lost in transit, so only 11 populations were represented in F2. Seeds were scarified with 150-grit sandpaper, soaked for 24 hours in tap water, and planted in 2-in x 8-in “cone-tainers” (Stuewe & Sons, Tangent, OR, USA). We sowed 5-8 seeds per pot in a 50-50 mixture of sterilized potting soil and sand and watered all pots to saturation for the first 14 days to ensure germination. The planting substrate had previously been sterilized via an autoclave at 121 C for 3 consecutive days. The greenhouse was maintained between 64-85 F under ambient light conditions from April to August 2023. No fertilizer was applied over the course of the experiment. On day 14, we thinned to a single individual per pot and randomly assigned plants to a watering treatment. The final design comprised of: 11 maternal populations X two F1 watering treatments X two F2 watering treatments X 48 individuals = 2112 plants, resulting in four treatment groups: DD (F1 dry / F2 dry), DC (F1 dry / F2 control), CD (F1 control / F2 dry), and CC (F1 control / F2 control) (Figure 1).

To ensure no control treatment plants experienced drought in the F2 generation, watering amounts were equal to the 30-year mean spring (March-June) rainfall amounts for the wettest seed source location in our study (60ml/week). To ensure all plants in the dry treatment group experienced water stress, we watered at a rate of 50% of the 30-year mean spring rainfall amount for the driest location in our study (15ml/week) (PRISM Climate Group). Plant stress was initially very high, so drought treatment was increased to 30ml/week on day 18 to ensure survival of the treatment group. Watering occurred twice per week at 30 ml (dry) and 60 ml (control) per watering using a syringe. Treatments began on day 14 and ended after the plants had fully gone to seed.

***FIGURE 1. map of 12 populations, experimental design, graph of precip/temp/CV***

***A screenshot of a computer

Description automatically generated***

*Trait data collection*

To investigate the transgenerational ef fects of drought in *P. patagonica*, we quantified the F2 generation’s germination rates, growth rate, mortality, time to flowering, biomass allocation, SLA, seed number, and seed weight in response to multigenerational dry conditions. Final height was determined as the maximum vegetative tissue height. We calculated relative growth rate with the height data, using the following formula (Crawley, 2009):

RGR = ln (final size / initial size)

Plant germination was monitored daily for 20 days. Plant height was measured every seven days for four weeks, and then every 14 days for four weeks. Plant mortality, presence of reproductive structures, and number of reproductive structures was monitored daily throughout the term of the experiment, from May 18th, 2023 to August 10th, 2023. Here, mortality rates represent proportion of plants alive on day 50, before most plants started flowering. On day 60, we collected one to five mature, healthy leaves per individual to measure SLA. Collected leaves were stored in ziplock bags with one water-saturated paper towel sheet and placed overnight in cool, dark conditions (MODEL AND TEMP OF FRIDGE). After 12-14 hours, leaves were weighed with a Mettler Toledo MODEL microbalance (1-μg precision; Mettler Toledo) to obtain saturated weight. The leaves were then scanned using a LEAF SCANNER MODEL (CITE) to assess leaf area. Leaves were dried for 48 hours in an oven at 60 C and then weighed again using the Mettler Toledo microbalance. Specific leaf area (SLA) was calculated as the area of fresh leaf divided by the oven dried mass. Leaf dry matter content was calculated as the leaf dry weight divided by the saturated leaf weight.

After each plant had finished flowering and the seed heads had begun to dry out but before the plant had senesced, seed heads were collected and stored for drying. Aboveground, belowground, and total biomass was collected immediately after seed collection and biomass allocation was calculated as the root:shoot ratio. The mass of the removed leaves was added back to the total aboveground biomass for each individual for analysis. The collected seedheads were placed in paper envelopes and dried for 3 months in a temperature-controlled laboratory room. In the lab, we cleaned the seedheads, collected 10-25 seeds per plant, and weighed them on a Mettler Toledo MODEL microbalance (1-μg precision; Mettler Toledo) to obtain the mean individual seed mass per plant. We then weighed the total seed from each plant to obtain total seed mass per plant. We used these measurements to calculate total seed number using the following formula:

total number of seed per plant = (n number of seeds weighed \* total seed weight of plant) / (weight of n seed)

*Statistical analysis*

1. *Is there evidence of transgenerational plasticity in response to drought in P. patagonica?*

To assess the effects of offspring (OT) and parental (PT) watering treatments on the phenotypic expression of functional and life history traits and whether these expressions differed between populations, we fit mixed effect models on each trait using the packages *lme4* and *nlme* (Bates et al., 2015; Pinheiro et al., 2023). We analyzed the plant response to water availability using the following response variables: 1) shoot biomass 2) root biomass 3) total biomass 4) root:shoot ratio 5) days to flowering 6) number of seedheads 7) RGR 8) final height 9) mortality 10) SLA 11) LDMC 12) seed mass and 13) seed number.

For the continuous variables (aboveground biomass, belowground biomass, total biomass, root:shoot ratio, RGR, maximum height, SLA, LDMC, and seed mass), binomial data (mortality status, flowering status), and count data (days to flowering, number of seedheads, seed number), we used Gaussian, binomial, and Poisson error structures, respectively. Our initial models included the parental watering treatment (PT), offspring watering treatment (OT), population, and all possible interactions as fixed effect. Tray was included as a random effect to account for greenhouse effects. The significance of fixed effect factors was assessed using the function *Anova* with a type III sum of squares test to calculate the residual degrees of freedom (package *car*, Fox & Weisberg, 2019). To assess model fit, marginal R2 (variance explained by fixed factors) and conditional R2 (total variance explained) were calculated using *r.squaredGLMM* (package *MuMIn*, Bartoń 2023).

For our generalized linear models (mortality status and flowering status), the random effect of tray was removed due to convergence issues. The significance of fixed effect factors was assessed using the function *Anova* with a type III sum of squares test. To assess model fit, McFadden’s pseudo R2 was calculated. For our generalized mixed effect models examining flower data (days to flowering, number of seedheads, and seed number), the significance of fixed effect factors was assessed using the function Anova with a type III Wald chisquare test. To assess model fit, marginal R2 (variance explained by fixed factors) and conditional R2 (total variance explained) were calculated using *r.squaredGLMM.*

We verified assumptions of independence and normality of residuals by plotting residuals from each model. When necessary, we specified the appropriate covariance structure to stabilize heteroscedascity between population responses using the *weights* argument in the function *lme* from the *nlme* package. Additionally, if transforming the response variable improved linearity and normality of the residuals, we used the transformed response variable. Root:shoot ratio, SLA, and LDMC were logged, and seed mass was square rooted.

A significant effect of OT meant differences between plants grown in the two watering treatments (drought or control), e.g. within-generation plasticity. A significant effect of PT means there are phenotypic differences between plant grown from seeds produced under different parental treatments of watering, e.g. transgenerational plasticity. A significant effect of the OT x PT interaction indicates that the effect of parental treatment on the phenotype depended on the offspring watering treatment (non-parallel norms of reaction) (Table 2).

1. *Is the initial seed source site VPD driving the transgenerational response to drought?*

When the PT x population, OT x population, or OT x PT x population term was significant, indicating that the within generation or transgenerational plasticity varied across populations, we performed additional analyses for each population to test if the trait response was associated with the climate of origin to elucidate any mechanism of TGP. Specifically, we tested if the climate at origin VPD influenced the expression of transgenerational plasticity in response to drought. To assess the effects of offspring (OT) and parental (PT) watering treatments, and climate at origin VPD on the phenotypic expression of functional and life history traits across generations, we fit mixed effect models for each trait that was significant in our initial models using the packages *lme4* and *nlme* (Bates et al., 2015; Pinheiro et al., 2023) (Table 2). Here, we analyzed plant response to multiple generations of drought using the following response variables: 1) root biomass 2) shoot biomass 3) total biomass 4) root:shoot ratio 5) final height 6) relative growth rate 7) SLA 8) LDMC 9) mortality status 10) flowering status 11) days to flowering and 12) seed number. Here, our models included the parental watering treatment (PT), offspring watering treatment (OT), growing season VPD (March-June) of initial collection site, and all possible interactions as fixed effect. Tray was included as a random effect to account for greenhouse effects. We used the same statistical approach as detailed above.

1. *Is the transgenerational response to drought adaptive?*

To test whether transgenerational plasticity was adaptive, we calculated the plasticity between treatments CC – DD using the relative distance plasticity index (RDPI) for traits related to performance (total biomass, root:shoot ratio) using the *rdpi* function in the *plasticity* package (Valladares et al., 2006, Ameztegui 2017). We then calculated Pearson correlations between trait plasticity and four traits related to fitness (seed number, seed mass, mortality status, and flowering status).

All analyses were performed using R Statistical Software v4.3.1 (R Core Team 2023).

## Results

*Transgenerational plasticity in response to drought*

We found moderate phenotypic differences in several traits affected by parental drought (Table 2; term PT), confirming there was some transgenerational plasticity in response to drought in *P. patagonica*. Offspring from parents that experienced drought generally showed an overall 4% decrease in SLA values compared to offspring from parents who experienced well-watered conditions (significant PT effect, F = 4.8770, P = 0.026; Figure 1D; see Supporting Information—Table S1). More specifically, plants that experienced two generations of drought (DD) experienced no change in SLA from control (CC), but plants that experienced one parental generation of drought and one offspring generation of control watering (DC) had the lowest SLA value, while plants that did not experience a parental generation of drought but did experience it in the offspring generation (CD) had the highest SLA values. The reduction in SLA only in the DC treatment rather than the DD treatment suggests that the plants in the DC treatment may be more efficient in water-use than plants with two generations of drought, suggesting that there may be a priming effect to drought, rather than an accumulation of adaptation to drought over multiple generations.

Additionally, offspring from parents that experienced drought generally showed an overall 47% increase in the number of plants that did go to flower (significant PT effect, F = 4.9721, P = 0.02596; Figure 1F; see Supporting Information—Table S1). Similarly to above, plants from the DD treatment group had lower flowering rates than plants that experienced the control treatment (CC), but again, plants from the DC group had the highest flowering rate, higher than both DD and CC. Plants that only experienced drought in the offspring generation (CD) had the lowest flowering rates of all. Like the patterns in SLA, this suggests that plants in the DC treatment were more adapted to drought than the DD treatment, suggesting the mechanism of priming.

However, more often, the effect of parental treatment depended on seed source population, indicating population-level variation in transgenerational effects of drought (pop x PT interaction in Table 2; Figure 2; see Supporting Information – Figure S1). We only found significant effects of parental treatment on SLA and flowering status (Figure 1D, 1F), but we found significant population by parental treatment interactions for shoot and total biomass, maximum vegetative height, SLA and LDMC, and mortality rate, and a marginal population by parental treatment interactions for root biomass and total number of seeds (significant pop x PT effect; Table 2; see Supporting Information – Figure S1). Additionally, we found several significant three-way interactions between offspring treatment, parental treatment, and population effects for shoot and total biomass, SLA, and mortality rate. When we found a significant two-way or three-way interaction that included PT, we passed the traits onto our seed source site VPD – TGP model, and these results will be discussed further as they related to seed source site VPD rather than population.

*Within generation plasticity in response to drought*

We found stronger phenotypic differences in traits affected by offspring drought (Table 2; term OT), indicating that within-generational plasticity in response to drought was very strong in *P. patagonica,* especially in traits related to aboveground biomass. The phenotypic expression of plants for most traits was significantly affected by the offspring drought treatment, especially in shoot biomass (significant OT effect, F = 41.52508, P = <.0001; Figure 1B) and R:S ratio (significant OT effect, F = 32.7771, P = <.0001; Figure 1C). Here, we saw a large 63% reduction in aboveground biomass and a 39% increase in R:S ratio when the plants were exposed to drought in the offspring generation, compared to plants who experienced control watering conditions in the offspring generation (Figure 1B and 1C; see Supporting Information – Table S1).

Total biomass was also affected by offspring drought treatment, but not as strongly as shoot biomass or R:S ratio (significant OT effect, F = 9.4233, P = 0.0045, Figure 1A). Offspring that experienced drought conditions experienced a 45% decrease in total biomass compared to offspring who experienced well-watered conditions (Figure 1A). Several traits had marginally significant responses to offspring drought treatment, including LDMC (F = 3.0178, P = 0.08355), total number of seeds produced (X2 = 3.0500, P = 0.08134), and days to flower (X2 = 3.2720, P = 0.08484) (Table 2). Offspring that experienced drought generally showed an overall 16% increase in LDMC values, 57% decrease in seed number, and 8% increase in days to flower compared to offspring who experienced well-watered conditions (Figure 1E, 1H, 1G). Additionally, most of the traits discussed above (root, shoot, and total biomass, R:S ratio, maximum vegetative height, SLA and LDMC, and mortality rate) also had a significant effect of pop x OT, indicating substantial within-generation plasticity between populations in trait responses (Table 2; see Supporting Information – Figure S1).

*Home site VPD and transgenerational responses*

For the traits that had a significant effect of PT, pop, or interactions that included PT or pop, we passed the traits onto our seed source site VPD – TGP model to evaluate whether transgenerational effects were related to the 30-year mean annual spring VPDmax (kPa) of the site where seeds were initially collected. Plants whose home site VPD was higher were from areas with stronger atmospheric drought and therefore a more stressful environment, while plants from lower home site VPD experienced weaker atmospheric drought, and therefore less stressful environments.

For several traits, we found moderate phenotypic differences in traits from plants sourced from different VPDs, regardless of offspring or parental treatment. R:S ratio (F = 6.9288, P = 0.008674) and flowering rate (F = 18.0738, P = 2.125e-05) both had a significant effect of VPD, while maximum vegetative height (F = 3.25561, P = 0.0715), SLA (F = 2.8156, P = 0.09404) and mortality rate (X2 = 2.9057, P = 0.088323) had marginally significant effects of VPD (significant VPD effect; Table 3; see Supporting Information – Figure S2). This suggests that local adaptation may be at play, where plants are phenotypically different based on seed collection site environment, even after two generations away from their home environment.

Shoot biomass (F = 5.55986, P = 0.0187) had a significant interactive effect of offspring treatment and VPD, indicating that within-generation plasticity of shoot biomass changed based on home site VPD, even two generations removed from home site (significant OT x VPD effect; Table 3; see Supporting Information – Figure S3).

We found moderate evidence for transgenerational plasticity mediated by home site VPD, particularly in days to flower (significant OT x PT x VPD term; X2 = 3.9065, P = 0.04810). Here, we found that the response of the offspring to drought varies depending on the conditions experienced by the parent plant, and the offspring response is modulated by home site VPD (Figure 2). Plants whose home site VPD was higher (=stronger atmospheric drought, more stressful environment) took longer to flower under two generations of drought, while plants from lower home site VPD (=weaker atmospheric drought, less stressful environment) had a shorter day to flower period under two generations of drought (DD; Figure 2). Similar to the trends above in SLA and flowering rate, plants that experienced one parental generation of drought and one offspring generation of control conditions (DC) overall took a shorter amount of time to flower than control conditions, and plants in this treatment group whose home site VPD was more stressful/higher took the shortest amount of time to flower. (DC; Figure 2). Plants who only experienced drought in the offspring generation, the least ‘prepared’ for drought, did not have any plants flower in populations from the lowest VPD nor populations from the highest VPD (CD; Figure 2).

*Adaptive transgenerational plasticity*

To assess the adaptive capacity of the TGP response, we calculated Pearson correlation coefficients between plasticity of traits related to performance (total biomass, R:S ratio) and traits related to fitness in annual plants (seed number, seed mass, mortality rate, flowering rate). We found several significant performance plasticity – fitness correlations. Total biomass plasticity was weakly positively correlated with proportion of plants flowering (R = 0.141, P = 0.0007192; Table 4). This suggests that under drought, as total biomass plasticity increases, more plants flower. Additionally, R:S ratio plasticity was weakly positively correlated with proportion of plants alive (R = 0.111, P = 0.007845) and more strongly negatively correlated with proportion of plants flowering (R = -0.297, P = 4.449e-13; Table 4), indicating that under drought, as R:S ratio plasticity increases, mortality rates decrease but fewer plants flower. **When relating trait plasticity to home site climate, we found a very weak but significant negative correlation between R:S ratio plasticity and home site VPD (R = -0.0729, P = 0.01808). Additionally, we found a stronger negative correlation between total biomass plasticity and home site VPD (R = -0.174, P = 1.279e-08).**

**Table 2**. Results of mixed models testing the effects of offspring watering treatment (OT), parental watering treatment (PT), population and their interactions on traits in *Plantago patagonica*. P values = p. < 0.1; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001. Signiﬁcant terms are shown in bold; terms in italics are marginally signiﬁcant.

1. F-statistic, signiﬁcance levels and degrees of freedom (d.f.) are shown for each term. Greenhouse tray was included as a random factor. R2m = marginal R2 ; R2c = conditional R2.
2. F-statistic, significance levels, and degrees of freedom (d.f.) are shown for each term. R2 = MacFayden’s pseudo R2.
3. Chi square-statistic, significance levels, and degrees of freedom (d.f.) are shown for each term. R2m = marginal R2 ; R2c = conditional R2.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| a) linear mixed effect models | | | | | | | | | |
| F-statistic, signiﬁcance levels and degrees of freedom (df) are shown for each term. Greenhouse tray was included as a random factor. R2m = marginal R2 ; R2c = conditional R2. | | | | | | | | | |
|  | **OT** | **PT** | **pop** | **OT x PT** | **pop x OT** | **pop x PT** | **pop x OT x PT** |  |  |
| **trait** | F | F | F | F | F | F | F | R2m | R2c |
| **root biomass** | 1.48898 | 1.72659 | **2.23247\*** | 0.87408 | **2.31735\*** | *1.81601#* | *1.75095#* | 0.193 | 0.310 |
| **shoot biomass** | **41.52508\*\*\*** | 0.78124 | **5.4445\*\*\*** | 0.26207 | **3.75759\*\*\*** | **4.59526\*\*\*** | **4.11189\*\*\*** | 0.690 | 0.813 |
| **total biomass** | **9.42335\*\*** | 2.02115 | **3.01112\*\*\*** | 0.81722 | **1.85775\*** | **2.10403\*** | **1.88905\*** | 0.289 | 0.416 |
| **R:S ratio** | **32.7771\*\*\*** | 0.32597 | **3.64578\*\*\*** | 0.36123 | **3.78680\*\*\*** | 1.41482 | 1.17273 | 0.218 | 0.282 |
| **max height** | 0.02087 | 0.32282 | **8.00415\*\*\*** | 0.11412 | **2.41572\*\*** | **2.31192\*** | 1.08794 | 0.118 | 0.226 |
| **RGR** | 1.70183 | 0.00163 | **3.26459\*\*\*** | 0.76076 | 1.58842 | 1.44775 | 1.27271 | 0.107 | 0.178 |
| **SLA** | 0.13450 | **4.9770\*** | **3.1309\*\*\*** | 1.09060 | **2.2754\*** | **2.2168\*** | **2.0565\*** | 0.172 | 0.237 |
| **LDMC** | *3.0178#* | 1.24810 | 1.34390 | 0.20180 | **2.1852\*** | **2.1772\*** | 1.53610 | 0.134 | 0.352 |
| **seed mass** | 0.95980 | 0.71500 | 1.51770 | 0.06010 | 0.54030 | 0.89400 | 0.04420 | 0.253 | 0.469 |
| df | 1 | 1 | 10 | 1 | 10 | 10 | 10 |  |  |
| b) generalized linear model | | | | | | | | | |
| F-statistic, significance levels, and degrees of freedom (df) are shown for each term. R2 = MacFayden’s pseudo R2. | | | | | | | | | |
|  | **OT** | **PT** | **pop** | **OT x PT** | **pop x OT** | **pop x PT** | **pop x OT x PT** |  |  |
| **trait** | F | F | F | F | F | F | F |  | R2 |
| **mortality status** | 0.7134 | 0.0497 | **2.5874\*\*** | 0.5717 | **1.8834\*** | **1.8456\*** | **1.9656\*** |  | 0.110 |
| **flowering status** | 1.7941 | **4.9721\*** | **12.3553\*\*\*** | 0.6823 | 1.1064 | **3.9357\*\*\*** | 1.1124 |  | 0.302 |
| df | 1 | 1 | 10 | 1 | 10 | 10 | 10 |  |  |
| c) generalized linear mixed effect model | | | | | | | | | |
| Chi square-statistic, significance levels, and degrees of freedom (df) are shown for each term. R2m = marginal R2 ; R2c = conditional R2. | | | | | | | | | |
|  | **OT** | **PT** | **pop** | **OT x PT** | **pop x OT** | **pop x PT** | **pop x OT x PT** |  |  |
| **trait** | X2 | X2 | X2 | X2 | X2 | X2 |  | R2m | R2c |
| **seed number** | *3.0379#* | 0.0952 | **20.1286\*** | 0.0664 | 12.4509 | *15.3847#* | 0.1102 | 0.397 | 0.576 |
| **number of seedheads** | 0.0292 | 1.6037 | 8.2482 | 0.1137 | 2.4939 | 4.2402 | 0.0551 | 0.130 | 0.183 |
| **days to flower** | *2.9696#* | 0.1144 | **22.3392\*** | 2.2929 | 5.3353 | 12.1751 | 4.2936 | 0.206 | 0.345 |
| df | 1 | 1 | 10 | 1 | 8 | 9 | 2 |  |  |

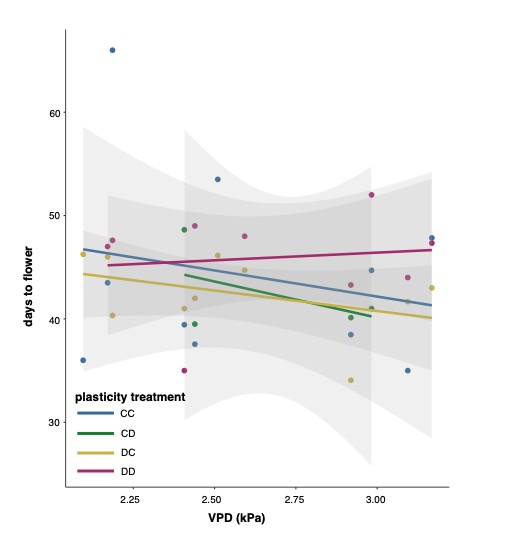
**Figure 1.** Effect of parental (PT) and offspring (OT) treatments on functional traits in *P. patagonica:* a) total biomass; b) shoot biomass; c) root:shoot ratio; d) specific leaf area (SLA); e) leaf dry matter content (LDMC); f) flowering rate; g) days to flower; h) total number of seeds produced. Each line shows the norm of reaction for a trait for offspring of control watering (blue) vs. drought watering (red) maternal plants. Observed means +- SE for 11 populations, with 4-12 replicates per population and treatment, are represented. Significance levels: ns= not significant, \* = P < 0.05; \*\* = P < 0.01; \*\*\* = P < 0.001. pop: population, OT: offspring treatment, PT: parental treatment. An interaction between pop and OT or PT indicates population variation for plasticity. A significant interaction with OT indicates within generational plasticity, and a significant interaction with PT indicates transgenerational plasticity. See Table 2 for full results.

**Table 3**. Results of mixed models testing the effects of offspring watering treatment (OT), parental watering treatment (PT), and seed source climate at origin value: the 30-year mean annual spring VPDmax (kPa) for each seed source location. Traits were only tested if population or an interaction with population was a significant fixed effect in Table 1. P values = p. < 0.1; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001. Signiﬁcant terms are shown in bold; terms in italics are marginally signiﬁcant.

1. F-statistic, signiﬁcance levels and degrees of freedom (d.f.) are shown for each term. Greenhouse tray was included as a random factor. R2m = marginal R2 ; R2c = conditional R2.
2. Chi square-statistic, significance levels, and degrees of freedom (d.f.) are shown for each term. R2m = marginal R2 ; R2c = conditional R2.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| a) linear mixed effect models | | | | | | | | | |
| F-statistic, signiﬁcance levels and degrees of freedom (df) are shown for each term. Greenhouse tray was included as a random factor. R2m = marginal R2 ; R2c = conditional R2. | | | | | | | | | |
|  | **OT** | **PT** | **VPD** | **OT x VPD** | **PT x VPD** | **OT x PT** | **OT x PT x VPD** |  |  |
| **trait** | F | F | F | F | F | F | F | R2m | R2c |
| **root biomass** | 0.16110 | 1.67670 | 0.25350 | 0.35700 | 1.35870 | 0.74340 | 0.68730 | 0.136 | 0.263 |
| **shoot biomass** | **17.49323\*\*\*** | 0.12514 | **12.2096\*\*\*** | **5.55986\*** | 0.04693 | 0.58477 | 0.39041 | 0.620 | 0.827 |
| **total biomass** | 1.9712 | 1.2413 | 2.5574 | 0.0000 | 0.9224 | 0.5214 | 0.4535 | 0.231 | 0.382 |
| **R:S** | *3.5623#* | 0.2609 | **6.9288\*\*** | 1.3204 | 0.1632 | 0.0173 | 0.0060 | 0.088 | 0.142 |
| **max height** | 2.2429 | 0.0303 | *3.25561#* | 1.8523 | 0.1221 | 0.5791 | 0.4070 | 0.030 | 0.217 |
| **RGR** | *4.0290#* | 0.0839 | 0.6758 | 2.1407 | 0.0707 | 0.4583 | 0.5765 | 0.027 | 0.107 |
| **SLA** | 0.9394 | 0.2146 | *2.8156#* | 0.6825 | 0.1441 | 0.0000 | 0.0001 | 0.035 | 0.096 |
| **LDMC** | 1.4117 | 0.0094 | 0.4045 | 0.6771 | 0.0969 | 1.9643 | 1.6973 | 0.038 | 0.285 |
| df | 1 | 1 | 1 | 1 | 1 | 1 | 1 |  |  |
| b) generalized linear mixed effect model | | | | | | | | | |
| Chi square-statistic, significance levels, and degrees of freedom (df) are shown for each term. R2m = marginal R2 ; R2c = conditional R2. | | | | | | | | | |
|  | **OT** | **PT** | **VPD** | **OT x VPD** | **PT x VPD** | **OT x PT** | **OT x PT x VPD** |  |  |
| **trait** | X2 | X2 | X2 | X2 | X2 | X2 | X2 | R2m | R2c |
| **mortality status** | 0.8134 | 1.8349 | *2.9057#* | 1.2008 | 1.4511 | 0.8761 | 0.7670 | 0.021 | 0.066 |
| **flowering status** | 0.3237 | 0.7222 | **18.0738\*\*\*** | 0.0078 | 0.2944 | 0.4581 | 0.5327 | 0.156 | 0.231 |
| **seed number** | 0.5442 | 0.4282 | 0.2634 | 0.2511 | 0.6728 | 0.9798 | 1.549 | 0.242 | 0.429 |
| **days to flower** | **3.9291\*** | 2.5837 | 0.1354 | *3.7991#* | *3.0365#* | *3.1938 #* | **3.9065\*** | 0.071 | 0.209 |
| df | 1 | 1 | 1 | 1 | 1 | 1 | 1 |  |  |

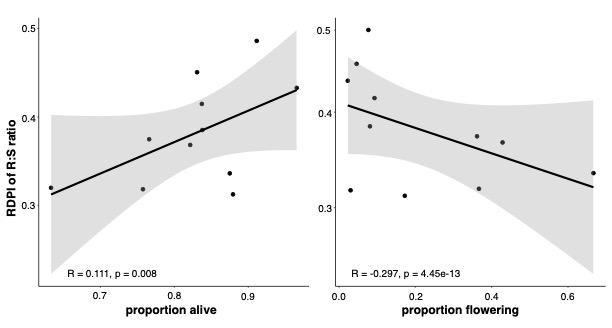
**Figure 2.** Effects of VPD of seed source origin and plasticity treatment on days to flower, the trait where the response of the offspring to parent treatment was dependent on seed source VPD. Grey areas represent SE.

****

**Table 4**. Results of linear regressions testing for relationships between fitness traits and transgenerational RDPI (CC-DD) of performance traits in response to drought. Shown are the R2-values. P values = p. < 0.1; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001. Signiﬁcant terms are shown in bold; terms in italics are marginally signiﬁcant.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Pearson's correlation coefficient and signiﬁcance are shown for each term. | | | | |
|  | **seed number** | **seed mass** | **proportion alive** | **proportion flowering** |
| **RDPI of total biomass** | -0.029 | -0.022 | -0.072 | **0.141**\*\*\* |
| **RDPI of R:S ratio** | -0.157 | -0.171 | **0.111\*\*\*** | **-0.297\*\*\*** |

**Figure 3.** Relationship between a) R:S trait plasticity across seed source environments and mortality rate and b) R:S trait plasticity across seed source environments and flowering rate for 11 populations of *P. patagonica*. Proportion alive was measured on day 50 The grey areas indicate 95% confidence intervals of the correlations.



maybe add climate correlations into the table and one more figure based on those correlations, either VPD or SAT

## Discussion

As climate change rapidly progresses, drought conditions and elevated aridity are an increasingly common phenomenon and can have profound consequence for the stability of ecosystems and the services they provide (Berdugo et al., 2020). To predict and manage biodiversity on planet earth, it is critical to understand the mechanisms that generate resilience to climate change. Transgenerational plasticity may be one mechanism by which species can withstand rapidly changing environments (J. Herman & Sultan, 2011). Here, we used 11 populations of *Plantago patagonica* sourced across a VPD gradient and exposed to fully factorial, multi-generation drought experiment, to ask: 1) is there evidence of transgenerational plasticity? 2) are transgenerational patterns related to home site atmospheric drought? and 3) is there evidence that transgenerational plasticity is adaptive? We found evidence of transgenerational plasticity in *P. patagonica* that suggests a shift towards a drought avoidance strategy, where *P. patagonica* plants from home sites with higher VPDs became more drought avoidant with increasing generations of drought exposure (Figure 2). Additionally, we found that plants with increasing plasticity in R:S ratio experienced higher survival rates but lower flowering rates under drought conditions, supporting our drought avoidance finding, but not necessarily enhancing fitness via transgenerational plasticity. Below, we discuss each of these results and their implications.

*Transgenerational plasticity in P. patagonica*

Annual plants like *P. patagonica* can employ two life strategies to deal with drought. Drought escape is associated with rapid growth, early flowering, and high leaf nitrogen level, where a plant escapes drought by reproducing and dying quickly, while drought avoidance is associated with high water use efficiency, lower stomatal conductance, denser leaves, and higher root:shoot ratios, where a plant invests more of its resources into avoiding the negative effects of drought (Kooyers, 2015). Here, we saw evidence for transgenerational plasticity (significant PT term; Table 2) in SLA and flowering rate for *P. patagonica*, which suggests that drought induced transgenerational cues shifts *P. patagonica* toward a drought avoidance strategy.

Plants that experienced parental drought treatments had lower SLA values, while plants that experienced parental control treatments had higher SLA values (Figure 1D; Table S1). Lower SLA values correspond with slower growth rates (Hunt & Cornelissen, 1997), slower leaf expansion rates (Poorter et al., 2009), and overall, a more conservative growth strategy. Here, in both the DD and DC treatments, SLA remained lower compared to parental control conditions. This may indicate that plants who experience drought in the parental generation retain cues to build thicker, more drought resilient leaves that can withstand desiccation to future drought. This contrasts with past transgenerational plasticity studies performed on short lived plants, where plants either experienced no effect of parent treatment on SLA (Matesanz et al., 2022) or saw an increase in SLA under parental drought treatment (Ramos-Muñoz et al., 2024). Additionally, a resurrection study performed by Christie et al. found no difference in SLA values between herbarium collected ancestor plants who had not experienced 10-years of intense drought, and field collected descendent plants, who had experienced 10-years of intense drought in the Colorado Platea region (Christie et al., 2023), suggesting that changes in SLA between generations are highly dependent on recent drought experiences, rather than cumulative drought effects over years, suggesting a highly plastic trait.

Conservative resource strategies are often associated with a higher ability to tolerate harsh conditions, but reduced competition (Midolo & Wellstein, 2020; Read et al., 2014). Contrary to this expectation, we also saw an increasing in flowering rate in plants that were exposed to drought in the parental generation (significant PT term; Table 2; Figure 1F). This acceleration in days to flower is in line with other studies, where plants exposed to either heat or drought often flowered earlier (Groot et al., 2017). However, despite the significant increase in the overall flowering rate of plants exposed to drought in the parental generation, it took plants from the DD treatment group far longer to flower than any other treatment group (Figure 1G) and they produced the smallest amount of seed per plant (Figure 1H; ; Table S1). DD plants also had the highest R:S ratio (Figure C; Table S1). Taken together, despite the higher rates of flowering in plants that experienced parental drought, the delay in flowering, reduced seed production, and higher R:S ratio suggests a shift towards drought avoidance, where plants engage in a more conservative resource strategy to persist under prolonged stress (Levitt, 1980).

Additionally, the high number of significant pop x PT term found in our initial models suggests that while transgenerational plasticity across all groups is only moderately influential, transgenerational responses are highly dependent on seed source site (Table 2, Figure 2).

In addition to transgenerational responses, within generation plastic responses to drought were strong in magnitude, particularly in biomass amounts. The drought treatment applied in this greenhouse experiment was applied early (7-10 days after germinating for most plants) and was very strong (50% reduction of water amount for the driest seed source location, so the water reduction was much higher for populations sourced from wetter areas).

Overall, we found that both transgenerational and within-generational plasticity in response to drought benefited *P. patagonica’s* performance traits. Drought in the offspring generation and population resulted in a smaller sized plant but higher in R:S ratio, and drought in the parental generation resulted in higher survival and tougher leaves. However, two generations of drought negatively influenced reproduction traits, mostly via parental effects. Two generations of drought resulted in smaller number of seeds produced per plant, where population and parental treatment had the strongest effect, and longer days to flower, where offspring treatment had a stronger effect.

*Transgenerational responses are context-dependent on home site VPD*

Interestingly, plants that experienced drought in the parental generation but control watering in the offspring generation (DC) had the highest performing reproductive traits of all the treatments. This is contrary to what we might expect, as transgenerational plasticity arises when the parental environment is correlated with the environment experienced by the offspring (Bonduriansky, 2021; Colicchio & Herman, 2020b; J. Marshall & Uller, 2007; Uller, 2008). Here, we see the highest reproductive performance in plants that experienced a mismatch in parent and offspring environment, but only when drought occurred in the parental generation (DC), and not when drought occurred in the offspring generation (CD, higher reproductive performance than DD but far lower than control conditions). This pattern may be explained by environmental characteristics at the seed source location, even after two generations away. Here, *P. patagonica* was sourced from a gradient of VPD amounts. Transgenerational plasticity may be favored over within-generation plastic responses if there is high temporal environment autocorrelation, so conversely, within generation adaptive responses may be favored if there is high temporal environment heterogeneity (Auge et al., 2017; Bonduriansky, 2021; Colicchio & Herman, 2020b; Donelson et al., 2018; J. J. Herman et al., 2014; J. Herman & Sultan, 2011). VPD captures the difference between the amount of moisture held by the air and the potential moisture holding capacity of the air, and therefore capturing the effects of both precipitation and temperature on water loss from vegetation. In the spring, Southwestern areas with climatologically higher VPD levels are also levels of high VPD variance, primarily driven by rapid temperature changes during the spring season (Seager et al., 2015). Thus, in higher VPD areas where some of our populations were sourced, selection may have favored adaptive within-generation plasticity over transgenerationally plastic responses if offspring cues are more accurate than parental ones for some populations. This may explain the relationship in Figure 2, where we see plants from higher VPDs who have experienced two generations of drought (DD) have a longer time to flower, indicating a move towards a drought escape strategy, while plants from the DC treatment group have an increasingly shorter period to flowering as VPD increases. Days to flowering time is an extremely sensitive transition that must be well timed, especially in annual plants, has high rates of heritability, and has been frequently studied in other transgenerational plasticity experiments ((Deng et al., 2021; Griffith & Watson, 2005; Matesanz et al., 2022; Ramos-Muñoz et al., 2024; Wolfe & Tonsor, 2014).

Additionally, this ‘release’ of resources in the DC group has also been seen in one other study, where grandparental and parental effects of mild heat stress were investigated in Arabidopsis thaliana. Here, Groot et al. found when plants (F1) experienced mild heat stress, their offspring (F2) were prepared for the same stress, but when it was no applied, the plants shifted their resource load to overprovision their offspring (2017). When comparing our results from the DC treatment, this suggests that drought exposure in the parental generation might act as an environmental trigger, preconditioning offspring to allocate resources in a way that enhances reproductive output. Both of these results align with a broader framework of phenotypic continuity across generations (as described by Badyaev & Uller, 2009), in which the offspring’s response is shaped not only by their immediate environment but also by environmental cues experienced by parent plants. This raises intriguing questions about how plants ‘anticipate’ environmental conditions across generations and allocate resources to maximize fitness based on parental, or grandparental, experiences, that have not yet been untangled.

*Relating performance plasticity and fitness robustness*

We found that R:S plasticity was weakly correlated with proportion of plants alive, and more strongly negatively correlated with proportion of plants flowering, indicating that under drought, as R:S plasticity increases, survival increases but fewer plants flower. Here, we see strong evidence that plants from more physiologically stressful sites move towards a drought avoidance strategy rather than drought escape: plants that invest in roots over shoots can potentially improve access to soil moisture, enhancing their chances of survival under drought stress, but this comes at the cost of reproduction, as the energy invested in root development reduces resources available for flowering and seed production. However, *P. patagonica* is a short-lived spring or winter annual that cannot usually persist past the first periods of summer or spring heat, dependent on location. This tradeoff may confer a decrease in fitness and a change in population dynamics, especially as areas of the Southwest become more arid (Williams et al., 2020). Plants with high plasticity in R:S ratio may maintain their populations through survival, but reduced flowering and seed production will lead to lower reproductive success and a slower rate of population growth.

*Relating performance plasticity and home site VPD*

Additionally, we found a weak but significant negative correlation between R:S ratio plasticity and home site VPD, suggesting that atmospheric drought at seed source locations might be an important factor for the evolution of transgenerational plasticity. Populations from sites with higher spring VPD showed reduced plasticity in their R:S ratio. This finding contrasts prior work in *P. patagonica*, where Klein & Mitchell found that populations sourced from sites with higher spring temperatures had higher R:S plasticity (2023). However, this finding is only in relation to within-generation plasticity; this may suggest that seeds sourced from more physiologically stressful sites have reduced capacity to pass along transgenerational cues, or perhaps the more physiologically stressful environments have already imposed strong directional selection towards a mean R:S ratio, resulting in less trait variation (Hoffmann & Hercus, 2000).

**To put somewhere because I think I need it**

If maternal environmental effects were related to higher seed mass produced in optimum conditions, offspring of maternal plants grown under well-watered conditions should have higher fitness in both offspring treatments, a pattern not found in our study. Additionally, we found no difference in seed mass in seeds collected from the parental generation (Table S3). This suggests that rather than seed-provisioning, other mechanisms may be at play to induce transgenerational plasticity, such as methylation of DNA or changes in hormones. Further studies are needed to elucidate the mechanisms behind transgenerational responses.

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