

**GENETIC AND ENVIRONMENTAL VARIATION IN LEAF TRAITS AND
PHYSIOLOGY OF *IPOMOPSIS***

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ABSTRACT

Selection that differs between environments may maintain genetic differences in traits between two closely related species. In a natural hybrid zone, this environment-mediated selection could also prevent hybrids from successfully occupying parental habitats. Environment-mediated selection can act on vegetative and physiological traits. Yet, the relationship between vegetative traits and physiological traits to hybrid fitness has not been described for many plant systems. In this study, we investigated *Ipomopsis aggregata* and *Ipomopsis tenuituba* and their hybrids in different environments, which allowed us to determine if genetic, environmental, or genetic x environmental effects were acting on specific traits. We measured vegetative traits and physiological traits (rosette size, specific leaf area, leaf hair density, chlorophyll concentration and intrinsic water-use efficiency) on individuals of *Ipomopsis* that had been planted in a reciprocal transplant at three different elevations in Poverty Gulch, Gunnison County, CO. Two of the traits were affected significantly only by environment: rosette size at age 5 was lowest at the highest elevation site, and leaf hair density decreased with elevation. Intrinsic water-use efficiency showed only genetic effects in which F1 hybrids with *tenuituba* as the maternal parent (TA) had higher values than the reciprocal cross (AT). Specific leaf area and an index of chlorophyll concentration showed both genetic and environmental effects. Higher specific leaf areas were found at higher sites and *I. aggregata* had significantly higher SLAs than *I. tenuituba*. Chlorophyll concentration increased with elevation, and F₂ hybrids had concentrations significantly lower than any other genotypic class. No genetic x environment effects were found for any of the traits.

INTRODUCTION

Aspects of hybridization in plants have been extensively studied in order to better understand the process of speciation (Campbell 1997; Campbell 2003; Campbell et al. 2005; Hatfield and Schluter 1999; Ludwig et al 2004; Waser and Campbell 2004). Speciation requires both reproductive isolation and the divergence between two species (Waser and Campbell 2004). Speciation in flowering plants can be the result of two types of natural selection, one that is mediated by pollinators and the other that is driven by abiotic factors of the physical environment (Campbell 2003; Waser and Campbell 2004).

When natural selection is based on environmental conditions experienced during the vegetative growth of the plant, it can promote divergence in genetically based vegetative traits between species in different environments. If such divergent selection has driven speciation, it is predicted that hybrids between the two species will be less fit than the parental species in the environments of the parental species (Hatfield and Schluter 1999). In addition, the two species would now differ genetically in those traits that have experienced that divergent selection.

Understanding how hybrid plant zones work through environment mediated selection can help us understand the selection that led to the original speciation (Campbell 2003). Essentially, the fitness of hybrids can inform us about mechanisms of speciation (Campbell 2003). Hybrid fitness can vary with the environment, and this variation can be a result of physiological traits based on the genetics of the plant (Campbell et al. 2005). In some instances hybrids have reduced fitness as a result of genetic incompatibilities (Campbell and Waser 2001).

Only a few studies have examined how the vegetative and physiological traits of a

plant can influence hybridization (Ludwig et al. 2004; Campbell et al. 2005). Physiological differences between the species and hybrids that prevent hybrids from surviving or reproducing in the parental habitats would suggest that selection mediated by the environment is important in speciation. The main objective of this project was to determine the extent to which differences in physiological and vegetative traits between two species of *Ipomopsis* plants and their hybrids were genetic, environmental, or due to genetic x environmental interactions. In this study we aim to answer the question of how physiological and vegetative traits compare between plant types of different genetic backgrounds, including hybrids, and in different environments. Measuring the hybrids in addition to the parental species is informative because hybrids can be either intermediate or transgressive, depending on the underlying genetics of certain traits (Rieseberg et al. 1999). The information gathered in this study will complement a concurrent study of how these same traits affect the fitness of each species and of the hybrids in the different environments. In future research, this will then allow tests of divergent natural selection to be conducted, which will in turn help define the mechanisms of speciation for these species. The genetic basis of variation in vegetative traits has received relatively little attention (Ackerly et al. 2000). Furthermore little is known about the physiological aspects of plant performance that might explain variation in hybrid fitness (but see Campbell et al. 2005). In this study we examined growth rate, intrinsic water-use efficiency, specific leaf area, leaf hair density, and leaf chlorophyll concentration. Studies in other systems have suggested that all of these traits can affect aspects of plant performance (Ackerly et al. 2000, Richardson et al. 2001, Sims and Gamon 2002, Scheepens et al. 2010).

Understanding how these traits contribute to divergent natural selection allows us to better understand their role in controlling hybridization and learn about aspects of ecophysiology that are important to responding to changing climatic conditions. For instance, rosette size is a measurement of growth, which might affect survival and/or age at reproduction. Higher intrinsic water use efficiency can enhance the performance of plants in certain dry environments (Dudley 1996, Heschel and Riginos 2005). Additionally, specific leaf area can indicate ecophysiological characteristics like stress tolerance and leaf longevity, which can affect the survival of the plant (Scheepens et al. 2010). Leaf hair density, or trichome presence, is a trait that can function to protect the plant from UV damage, drought and temperature stress (Skaltsa et al. 1994, Perez-Estrada et al. 2000). Chlorophyll content is a primary factor in determining how much light energy can be captured to initiate the reactions of photosynthesis, which is ultimately the determinant of all plant functions and growth (Richardson et al. 2001). By investigating if these traits are affected by genetic or environmental effects we can then make inferences about the plasticity of the traits and how plants could respond to changing climate. In the future these traits can be correlated to fitness, and it can then be determined what role they play in the divergent natural selection of these two species.

METHODS

Natural History

These two species of *Ipomopsis* plants are primarily monocarpic and produce seed in the late summer. Seeds are gravity dispersed, and there is no seed bank. These characteristics allow experimental seedlings to be identified year after year without the problems of later seed development related to dormancy (Campbell 2003). Furthermore

because these species are monocarpic it is possible to measure their lifetime fitness by following each plant until its first and only flower production and therefore its single reproductive bout (Campbell 1997). *Ipomopsis* germinate in early summer, forming a small rosette of leaves, and spend 2-10 years in this vegetative state before blooming and dying (Campbell 1997). Phylogenetically *Ipomopsis aggregata* and *Ipomopsis tenuituba* are most likely each other's closest relatives, which indicates a recent speciation (Grant and Wilken 1986). *Ipomopsis* acts as a suitable specimen for these studies because of its natural hybridization, non-existent seed bank, short seed dispersal, and monocarpic status.

Study System

This study was conducted on pre-established reciprocal transplants in Poverty Gulch, Gunnison County, CO (USA). In this region, natural populations of *I. aggregata*, and *I. tenuituba* exist at low (up to 2,900 m) and high (at and above 3,200 m) elevations respectively (Campbell et al. 1997). A natural hybrid zone occurs at intermediate elevations between the two pure populations.

Poverty Gulch is a high mountain valley where the three experimental sites run up an elevation gradient. Starting at 2900 m (9,500 ft), the lowest site (latitude 38°57'33'' longitude 107°04'57'' latitude) is representative of where *I. aggregata* naturally occurs (hereafter *aggregata* site''). This is the wettest site and is on a relatively gentle slope at the bottom of the valley (Campbell and Waser 2001). The highest elevation site (38°59'01'' longitude 107°05'32'' latitude), where *I. tenuituba* naturally grows, is located on a steep and rocky slope at 3,200 m (10,500 ft) and tends to be drier than the *I. aggregata* site. A third site (latitude 38°57'67'' longitude 107°05'20'' latitude) is located between the two in the hybrid zone around 3,050 m (10,000 ft). This area, where natural hybrids are found, is actually the driest site, located on a steep talus slope with little vegetative cover (Wu and Campbell 2004).

Experimental Plantings and Setup

In 2007, the three sites were set up as a reciprocal transplant with 20 1x1 m blocks at each site. Experimental crosses were used to generate offspring of known genetic backgrounds that were planted at each site along with the parental species of *I. aggregata* (AA) and *I. tenuituba* (TT). Hand-pollinated crosses were performed in both directions, with *I. aggregata* as the female parent (AT; first letter refers to the mother), and *I. tenuituba* as the female parent (TA). These F₁ hybrids (AT, TA) were then crossed to create F₂ hybrids. The seeds of these five genetic classes (AA, TT, AT, TA, F₂ hybrids) were planted at 10 cm intervals in the 20 1x1 m blocks located in each of the three different environments. Blocks were established at least 1 m away from any naturally occurring flowering *Ipomopsis* plants to ensure that all germination was from the experimental study.

These sites represent different environmental conditions. In previous studies, the survival of reciprocal F₁ (TA vs AT) hybrids were different (Campbell and Waser 2001). F₁ hybrids showed dependence on the environment based on which species acted as the maternal parent (Campbell et al. 2005, Campbell and Waser 2001, Campbell et al. 2010). On average both F₁ hybrids survived as well as their parental species at each site, but in the sites of the parental species AT hybrids outperformed TA hybrids (Campbell and Waser 2001). This is most likely due to environment-dependent selection (Campbell and

Waser 2001). Furthermore Campbell et al. (2010) demonstrated that hybrids with *I. tenuituba* cytoplasmic genes had optimal photosynthesis at lower soil moisture than did reciprocal hybrids with *I. aggregata* cytoplasmic genes. In 2008 an additional 10 blocks were planted in the lowest elevation site in order to increase the sample size of F₂ hybrids. F₂ hybrids are expected to have a large diversity of trait values, making it easier to measure selection.

Sample Size

Vegetative and physiological leaf traits were measured along the elevation gradient at these three sites. During June 21st to June 26th, 2012, site censuses were conducted to determine individual survival from the previous summer, growth as a determinant of rosette size (described below), and presence of an inflorescence. These census data led us to sample 39 vegetative plants from the *I. aggregata* site, 36 vegetative plants from the hybrid site, and 76 rosettes from the *I. tenuituba* site.

Response Measurements: vegetative and physiological leaf traits

Rosette size and the leaf gas exchange needed to calculate water-use efficiency were measured on site for all vegetative rosettes at the three different elevation sites.

Rosette Size-During the initial census period, rosette size was determined in the field by measuring the length of longest leaf (mm) for each rosette and multiplying it by the total number of leaves. Since snowmelt exhibits a large effect on growing season (Galen and Stanton 1995), it was predicted that the highest elevation *tenuituba* site would produce smaller rosettes as a result of a shorter growing season.

$$\text{Rosette Size} = \text{Length of Longest Leaf} \times \text{Total Number of Leaves}$$

Water Use Efficiency-In order to gather the data associated with physiological traits, an infrared gas analyzer Li-Cor 6400 Portable Photosynthesis System was brought into the field. The Li-Cor measured photosynthetic rate (A) and stomatal conductance (g), which together yields intrinsic water-use efficiency (A/g). All measurements were made with saturating light conditions (PAR = 1800), a leaf temperature of 27°C, and a sample CO₂ concentration of 375ppm (Wu and Campbell 2007). *Ipomopsis* species tend to stop photosynthesizing in the early afternoon, so photosynthetic traits were measured between 9000 and 1400 hours (Wu and Campbell 2007). These physiological traits are strongly dependent on local moisture, and therefore soil moisture was also measured to use as a covariate in analyses if necessary. Previous research has shown that *I. aggregata* species from the wetter locations usually have a lower WUE, while hybrids, from the drier sites have a higher WUE (Wu and Campbell 2007, Campbell et al. 2010). Furthermore, TA F1 hybrids tend to have a higher WUE than the reciprocal F1 hybrid (AT) when measured in a single environment, although this difference has not yet been tested across all three sites (Wu and Campbell 2007, Campbell et al. 2010).

$$WUE = \frac{\text{Photosynthetic rate (A)}}{\text{Stomatal Conductance (g)}}$$

In order to measure specific leaf area, leaf hair density and chlorophyll content, single healthy green leaves were chosen randomly from each rosette and brought back to the lab for further analysis. Physical leaf traits of area, wet mass, dry mass, and reflectance spectrum were used to calculate the appropriate leaf traits.

Specific Leaf Area-In order to calculate leaf area, leaf samples were scanned and analyzed in ImageJ (NIH freeware). For dry mass, leaf samples were dried for 2 hours at 60°C in drying oven and then weighed individually with a balance to the nearest .0001 g. In some species, such as *Campanula thyrsoidea* (Scheepens et al. 2010), SLA decreases with increasing altitude, meaning the leaves will be thicker or have a higher leaf density or some combination of both. Due to the natural elevation gradient in this study system it was hypothesized that SLA could also follow this trend.

$$SLA = \frac{Area (cm^2)}{Dry mass (g)}$$

Leaf Hair Density-Scans from leaf area analysis were used (Appendix 1, Figure) and individual leaf hairs were counted manually off the scan in ImageJ. Leaf hair count was divided by the original area to get a measure of density. Based on other studies it was originally predicted that trichome production would be increased at higher and drier environments like the *tenuituba* and hybrid sites (Steets et al. 2010) .

$$LHD = \frac{Hair Count}{Area}$$

ChINDI-In order to estimate chlorophyll concentration, a leaf spectrum was taken on all individual leaves using a reflectance spectrometer at 400m-800m. An index was then used to determine chlorophyll content: $(R750-R705)/(R750+R705)$, where R indicates reflectance at a particular wavelength. This index is usually correlated with chlorophyll a concentration, which provides a basis for the amount of light energy a plant can absorb and then use for photosynthetic processes (Richardson et al. 2001, Sims and Gamon 2002). Plants at the high *I. tenuituba* site are less nitrogen-limited than the lower elevation sites (Campbell et al. 2010). Plants in a nitrogen deficient system would be expected to have a lower chlorophyll concentration because chlorophyll content is approximately proportional to leaf nitrogen content (Evans 1989).

$$ChINDI = \frac{(R750-R705)}{(R750+R705)}$$

Statistical Analysis

Each of the measured traits acts as a dependent variable and was compared against the two independent variables: environment (site location) and genetic class (AA, TT, AT, TA, or F₂). A 2-way ANOVA was used to analyze if there is variation among the plant genotypes, the site, and/or the interaction between genotype and site. In addition, the pure species AA and TT were compared using a priori contrast. The statistical tests were performed using the statistical software SAS (SAS Institute, Cary, NC). Because sample sizes were unequal, type III sums of squares were used for tests of main effects.

RESULTS

All results, with the exception of water-use efficiency for which the 2008 planting was also used to increase sample size, were based only on plant samples from the 2007 planting. Two of the traits were affected significantly only by environment: rosette size and leaf hair density (Table 1). Chlorophyll index and specific leaf area showed both genetic and environmental effects. Intrinsic water-use efficiency was the only trait that showed only genetic effects. There was no genetic x environment interaction detected for any of the traits. In other words, the effect of environment (site) on these traits did not depend on the genetic background of measured plants.

Rosette Size-Rosette size differed significantly by site ($F_{2,109}=3.49$, $P=.0410$), demonstrating an environmental effect. Surviving rosettes were only about half as big at the *tenuituba* site compared to the *aggregata* site (Figure 1).

Water–Use Efficiency-Water-use efficiency, $\mu\text{mol CO}_2/\text{mol H}_2\text{O (A/g)}$, differed between plant types ($F_{4,67}=2.76$, $P=0.0345$) (Figure 2), indicating a difference due to genotype class. The TA F1 hybrids had the highest water-use efficiency among all plant types. AT F1 hybrids had the lowest water-use efficiency.

Specific Leaf Area-An environmental effect was noticeable for specific leaf area ($F_{2,108}=4.230$, $P=0.0170$). SLA tended to increase with elevation (Figure 3). The *tenuituba* site had the highest SLA, and the *aggregata* site had the lowest SLA. While genotype was non significant overall, plant type AA did have a significantly higher SLA than TT based on a priori contrast ($F_{1,108}=5.14$, $P=0.0254$).

Leaf Hair Density-Similarly to rosette size, a significant environmental effect was found for leaf hair density ($F_{2,108}=3.92$, $P=0.0228$). Leaf hair density decreased with elevation and was lowest at the *tenuituba* site (Figure 4).

Chlorophyll a Concentration- Both genetic ($F_{4,109}=4.28$, $P=0.0030$) and environmental effects ($F_{2,109}=9.47$, $P=0.0002$) were seen for the index of chlorophyll a concentration (Fig. 5). On average chlorophyll concentration increased with elevation. The genetic effect was primarily due to a lower chlorophyll index in F_2 hybrids, compared to the average of all of the other genetic classes (a priori contrast $P=0.0006$).

DISCUSSION

Rosette Size-As expected, rosette size decreased with an increase in elevation (Figure 1). In this study rosette size is an indicator of plant performance based on growth. This means that wherever the most conducive environmental conditions exist the plant will be able to grow the most. Growth may be slowest at the highest elevation site because of the shorter growing season. It should be noted that in this experiment rosette size was measured only in year 5, and it is theoretically possible that plants at certain sites could have grown faster, bloomed and already died by this time. However, very few plants have bloomed at the *tenuituba* site to date, so the data do support a slower growth rate at the *tenuituba* site.

Water-Use Efficiency- Similar to previous studies, water-use efficiency differed among plants of different genetic backgrounds (Wu and Campbell 2007, Campbell et al. 2010). TA hybrids had the highest WUE, and this is consistent with prior research, which explains this variation by the cytoplasmic genes inherited maternally from *I. tenuituba*. The *I. tenuituba* and hybrid sites are drier (Wu and Campbell 2007), and plants adapted to drought often have a higher WUE (Heschel et al. 2004).

Specific Leaf Area- As for rosette size, the environment also played a role in the differences in SLA. SLA was higher at the higher sites, which is contrary to a previously observed pattern in the species *Campanula thyrsoidea*, where SLA decreased with increasing altitude (Scheepens et al. 2010). However while a genetic effect was non-significant overall, plant type AA did have a significantly higher SLA than TT based on a priori contrast. Scheepens et al. (2010) also reported a possible genetic effect due to adaptation to local conditions. In this *Ipomopsis* study SLA increased environmentally with elevation, but at the same time when planted at lower elevations the high elevation species of TT had a lower SLA than did AA. The environmental and genetic effects thus work in opposition, and the net result is that TT planted at the *tenuituba* site actually had a similar SLA to AA at the *aggregata* site (Figure 3). If the genetic effect had gone in the same direction as the environmental effect it would have been expected that TT have a higher SLA when planted at the *tenuituba* site than AA at the *aggregata* site. The results demonstrate how the genetic and environmental effects are working in opposition and essentially canceling each other out.

Leaf Hair Density- Differences in leaf hair density were also due to environmental effects. Leaf hair density decreased with elevation and was highest at the *aggregata* site. This is opposing to previous studies where leaf hair density increased at higher and drier sites like the *tenuituba* site (Steets et al. 2010). Higher leaf hair density at high elevation in other species could be explained by its role in UV protection, however, leaf hair density can also function as a protection agent against biotic stress like insects or pathogens (Agrawal et al. 2002, Handley et al. 2000). Species of *Ipomopsis* are subject to seed predation by *Hylemya* species (Brody 1992) and perhaps these insects were more prevalent at the *aggregata* site, as found by Campbell et al. (2002). *Hylemya* lay their eggs underneath the sepal of the flower (Campbell 1991), and correlations between hair density on the leaves and hair density on the calyx could help explain whether or not leaf hairs act as a defense to seed predators. Furthermore, in a previous year, it is interesting that TTs had significantly higher leaf hair density than AAs, which would demonstrate a genetic effect (D. Campbell, pers. comm.). It is possible that leaf hair density has both genetic and environmental effects. It is probable that the sample size prohibited any genetic effect from showing in 2012, and if there had been more TT samples at the *aggregata* site the possible genetic effect would have been detectable.

Chlorophyll Concentration- Our results demonstrated an environmental effect in that chlorophyll concentration increased with elevation. However genetic effects also contributed to the differences in chlorophyll concentration, with the parental species exhibiting higher chlorophyll concentrations than hybrids. Since the genetic effect was primarily due to low chlorophyll in the F₂ hybrids, these results indicate some hybrid breakdown in the second generation. During the formation of F₂ hybrids, new gene combinations are formed that may not work well together. In this instance, it is possible

that gene combinations in the F2 hybrids could be genetically insufficient at producing chlorophyll. On the other hand, *Ipomopsis* could be responding to a variety of environmental conditions. Differences in soil nutrients, like nitrogen, could have an effect on chlorophyll concentration. Previous studies with *Ipomopsis* demonstrate that nitrogen fertilization increased seed set at the *aggregata* and *hybrid* sites, but not the *tenuituba* site (Campbell et al. 2010). This could indicate that plants are nutrient limited at the lower sites because they responded by a growth increase with the addition of nitrogen. Photosynthetic rate is related linearly to leaf nitrogen (Evans 1989), which means that with a nitrogen deficiency, there could be less chlorophyll production. This nitrogen limitation at lower elevations could be a driving factor in lower chlorophyll concentrations.

CONCLUSIONS

Overall, this study allowed us to investigate the role genetic and environmental effects have on certain vegetative and physiological traits. The next step in this project is relating these vegetative and physiological traits to fitness in order to understand their role in divergent selection. While pollinator mediated selection also occurs in this system, the plant must survive in its vegetative stage before it can reproduce. This ultimately means that fitness effects can occur throughout the entire lifetime of the plant.

While this study is a rare representative of long term data associated with hybrid zones, the sample size for 2012 at the *aggregata* site was limited. With a larger sample size it is possible that the results from each of the traits would have been clearer to interpret. Furthermore, variation in yearly climatic conditions wasn't considered. However, long-term data is associated with this project, and, with time, past years can be combined with this data to see if trends are consistent throughout the years.

By understanding these traits and whether their differences are environmental or genetic we can also predict the plasticity of the trait and how fast it will change with a changing climate. In combination with relating these traits to fitness, we can make predictions about how well a plant can survive in a variation of environmental conditions. For traits like water-use efficiency where genetic effects appear dominant, if the climate changes rapidly, for example becoming drier, the population will only be able to respond over a time scale of evolutionary responses to natural selection. Because genetic by environment interactions were weak or non-existent, we could predict that the genetic differences seen currently (e.g. higher WUE for TA than AT hybrids) would also be maintained under a changed environment. On the other hand, traits with mainly environmental effects are more plastic and would change quickly with an environmental change.

In conclusion, this study established that both genetic and environmental effects account for the differences in vegetative and physiological trait values between hybrids and parental species.

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Table 1. Two-way analysis of variance (ANOVA) for environmental, genetic, and G x E, effects on vegetative and physiological traits in *Ipomopsis* species and hybrids grown in a reciprocal transplant under natural environmental conditions for each species.

Vegetative and Physiological Traits

Source of variance	Rosette Size		SLA		LHD		ChINDI		WUE	
	F	P	F	P	F	P	F	P	F	P
Site	F _{2,109} = 3.29	.00410	F _{2,108} = 4.23	.0170	F _{2,108} = 3.92	.0228	F _{2,109} = 9.47	.0002	F _{4,67} = 0.71	.4938
Plant type	F _{4,109} = 1.65	.1674	F _{4,108} = 1.67	.1625	F _{4,108} = 0.84	.5036	F _{4,109} = 4.28	.0030	F _{2,67} = 2.76	.0345
Site x Plant Type	F _{8,109} = 1.58	.1398	F _{8,108} = 0.67	.7155	F _{8,108} = 0.90	.5172	F _{8,109} = 1.87	.0714	F _{6,67} = 1.27	.2823

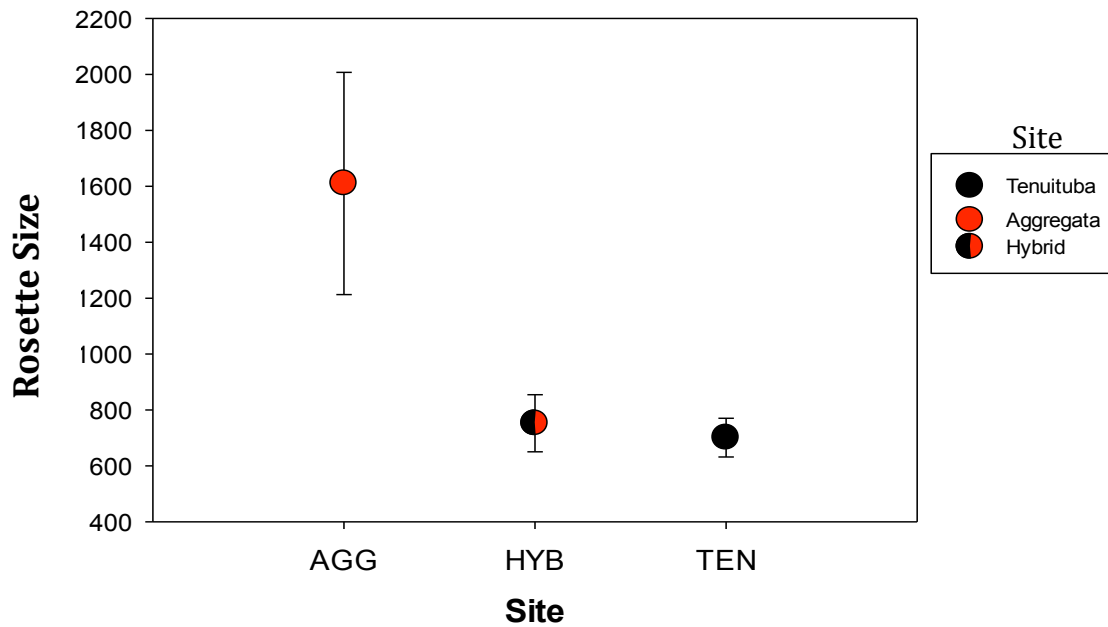


Figure 1. Means (+/-SE) of rosette size (longest leaf x number of total leaves averaged across the five genotypic classes of *Ipomopsis* species (AA, TT, AT, TA, F₂ hybrids) at sites where aggregata, hybrid, and tenuituba naturally occur.

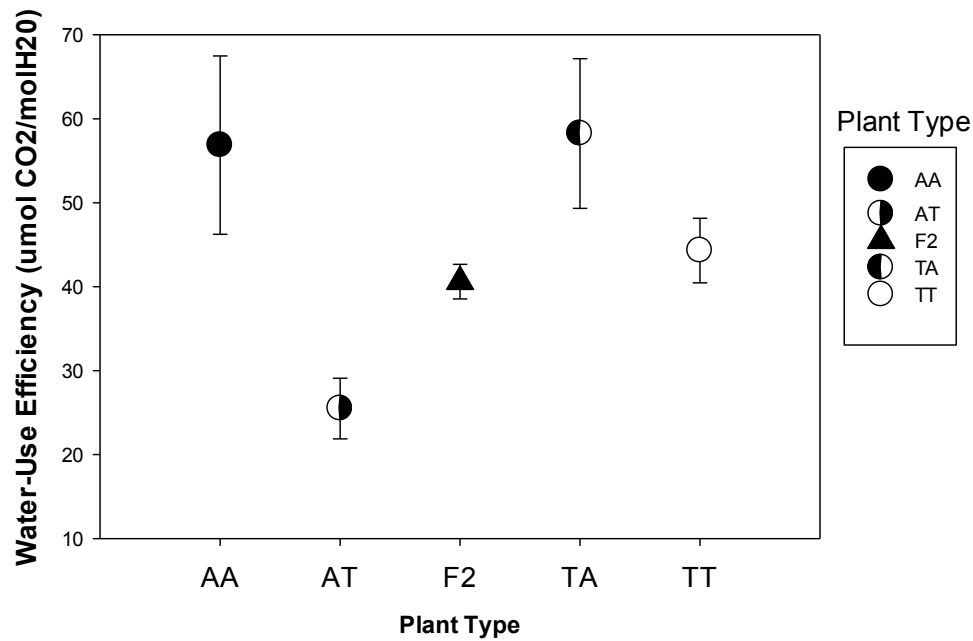


Figure 2. Means ($\pm$ SE) of intrinsic water-use efficiency ($\mu\text{mol CO}_2/\text{mol H}_2\text{O}$) for each of the five genetic classes of *Ipomopsis* species (AA, TT, AT, TA, F₂ hybrids) averaged across sites where aggregata, hybrid, and tenuituba naturally occur.

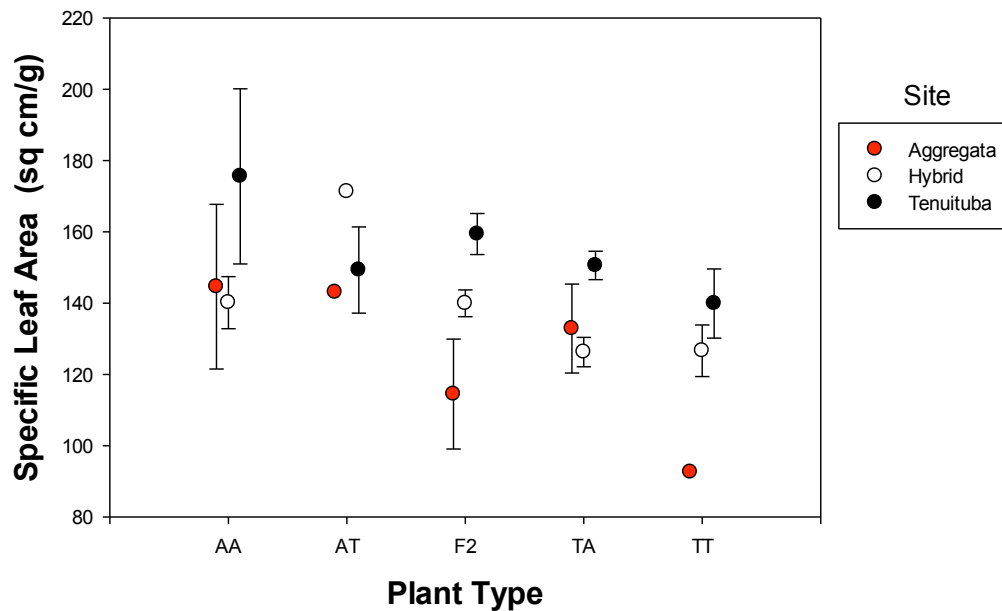


Figure 3. Means ($\pm$ SE) specific leaf area ($\text{cm}^2 \text{g}^{-1}$) for each of the five genetic classes of *Ipomopsis* species (AA, TT, AT, TA, F₂ hybrids) at sites where aggregata, hybrid, and tenuituba naturally occur.

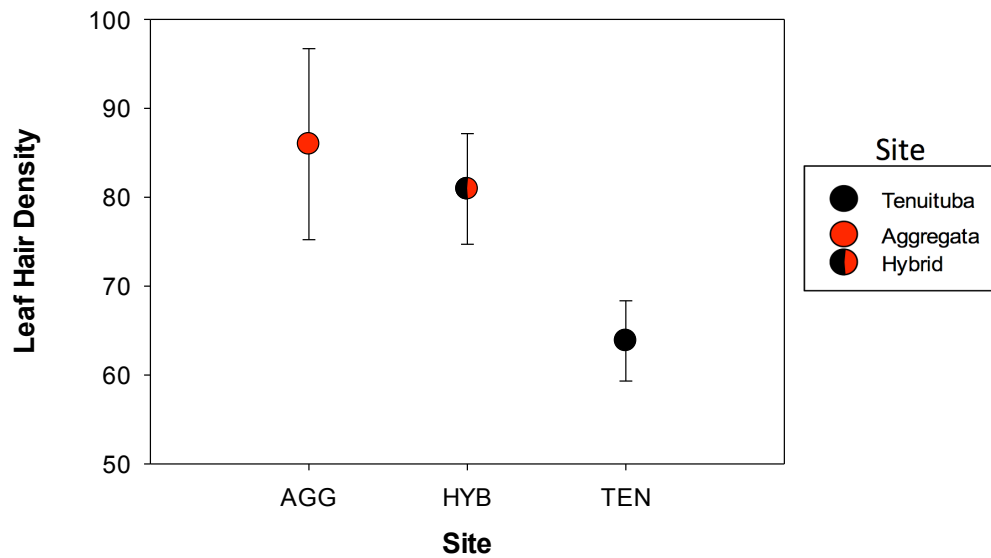


Figure 4. Means (+/-SE) of Leaf Hair density (hairs cm⁻²) averaged across the five genetic classes of *Ipomopsis* species (AA, TT, AT, TA, F₂ hybrids) at sites where aggregata, hybrid, and tenuituba naturally occur.

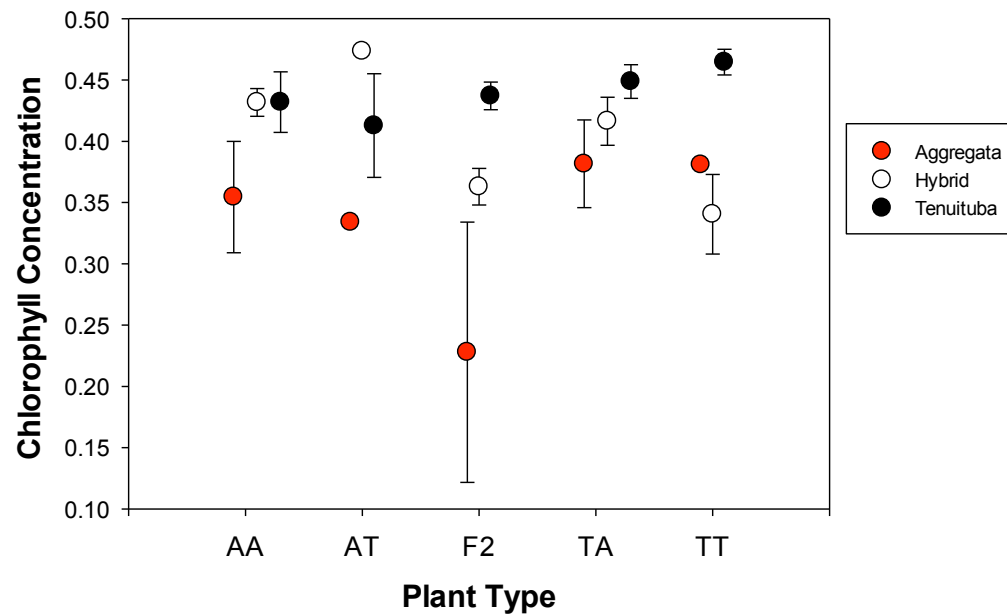


Figure 5. Means (+/-SE) of chlorophyll concentration for each of the five genetic classes of *Ipomopsis* species (AA, TT, AT, TA, F₂ hybrids) at sites where aggregata, hybrid, and tenuituba naturally occur.