Maternal Floral Color, UV Protection, and Germination in *Ipomopsis aggregata*

(Polemoniaceae)

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**Abstract**

Scarlet gilia (*Ipomopsis aggregata*) is a red to pink flowering plant which shows floral color plasticity within populations on the eastern slope of the Rocky Mountains. Anthocyanin is the red flavonoid pigment which gives these petals color and has been shown to act as protection from UV damage in plants by shielding chloroplasts and acting as an antioxidant. This study was conducted on seeds from red and pink flowering maternal plants at the Manitou Experimental Forest. After being counted and weighed, seeds were germinated in a growth chamber over the course of 23 days and were censused for germination rate every other day. Photosystem efficiency, anthocyanin content, chlorophyll content, and biomass were measured on germinated seedlings after the census was completed. Dark-flowering maternal plants yielded seeds and seedlings with higher biomass than light-flowering ones. Seeds from darker maternal plants also germinated faster than those from light maternal plants and had higher anthocyanin content. The hereditary nature of anthocyanin content thus suggests that higher anthocyanin levels are linked to measures of fitness such as higher seed weight, germination rate, and seedling biomass, indicating that UV protection provided by anthocyanins potentially increases the realized fitness of maternal plants.

**Introduction**

Plant reproduction centers around an adult individual's ability to produce nutritive and viable seeds in order to increase the likelihood of healthy progeny and thus individual fitness. The physiological relationship between maternal plants and their offspring depends on maternal genotype, seedling and adult environmental conditions, as well as maternal effects. Maternal fitness advantages are often most visible in germination success and early seedling physiology, positing these life history stages as fundamental drivers of realized fitness (Roach and Wulff

1987). An environmental condition which significantly affects these early life history stages is UV irradiance.

Ultraviolet radiation presents a threat to plant life history which continues to grow as climate change impacts global ecosystems. As temperatures and radiation levels rise, processes crucial for reproduction and fitness become misaligned, drastically changing the way plants interact with their environment. In terms of plant architecture, studies have shown UV damage to hinder both photosystem and stomatal function, as well as significantly alter canopy morphology in forests (Pandey and Pandey Rai 2014). Increasing UV radiation with climate change is associated with higher seasonal temperatures, which are correlated with lower seed viability and lower rates of germination (Perez and Kettner 2013). Lower seed viability and germination success under these environmental stressors often occur in conjunction with decrease in seed production and biomass under high levels of UV radiation (Teramura and Sullivan 1994). Increasing environmental stress on plant life history introduces evolutionary pressure for plants to adapt to variable UV and temperature levels. Populations of plants experiencing environmental pressure may also employ strategies outside of chromosomal contribution to impart stress tolerance capabilities to their offspring. These strategies often utilize other molecules in plant cells such as secondary metabolites and phytochromes to protect against oxidative damage from UV radiation. Particularly stressful UV conditions activate antioxidant defense systems, increasing secondary metabolite content to combat damage to critical plant morphology (Chen et al. 2019). Concentration within and sensitivity to these stress tolerance molecules can be products of phenotypic plasticity which influence evolutionary history (Heschel et al. 2014). Fluctuating light and temperature have been shown to impact different phytochromes in ways that can influence the early life history process of germination (Heschel et al. 2007, Yodpitaka et al.

2019). Specifically, higher levels of UV exposure have been shown to correlate with dark red petal color due to anthocyanin (Kawabata et al. 1999).

Anthocyanin is a red flavonoid secondary metabolite which has been shown to act as protection from UV damage in plants by shielding chloroplasts and acting as an antioxidant (Neill and Gould 2003). Anthocyanin content is also responsible for red pigmentation in many flowering plant species' petal color (Campitelli et al. 2017). Anthocyanin acts as an antioxidant by providing a photoprotective layer in vegetative tissue, physically shielding chloroplasts against oxidative damage and subsequent free radical production (Neil and Gould 2003, Steyn et al. 2002). High anthocyanin content has historically been correlated with increasing UV radiation levels, suggesting that anthocyanin content could be a plastic phenotypic trait which varies in response to UV stress (Pandey and Pandey-Rai 2014). There is also evidence to suggest anthocyanin plays a role in the process of germination, as in wheat plants higher anthocyanin content is correlated with faster rates of germination (Ahmed et al. 2009). Additionally, flavonoid pigments such as anthocyanin have been shown to be particularly valuable as UV protection during leaf production in rye, suggesting anthocyanin content may be important during seedling development (Burchard et al. 2000). Previous research posits anthocyanin as a possible mechanism of stress tolerance in plants experiencing high levels of UV radiation. Correlations between anthocyanin content and environmental factors such as light intensity, temperature, moisture, and pH imply that its involvement in stress tolerance is multi-factorial (Yang et al. 2017).

Because they are necessary for plant growth and reproduction, chlorophyll content and photosynthetic efficiency are functional traits which can indicate possible fitness levels later in a plant's life history (Heschel et al. 2007, Chen et al. 2019). UV radiation has been shown to

2

significantly affect the relative amounts of chlorophyll A and B in vegetative tissue, indicating that radiation has the ability to divert plant function from its carefully maintained equilibrium, and possibly decrease photosynthetic ability (Larsson et al. 1998). Chlorophyll is the green pigment in chloroplasts which, when bleached, can prevent proper chloroplast function. Chlorophyll content index (CCI) is representative of the amount of functional chloroplasts present within vegetative tissue. Functional chloroplasts are vital for plant health because photosynthesis occurs across their internal membrane. However, anthocyanins have been shown to prevent such bleaching in vegetative tissue (Neil and Gould 2003). If chloroplasts are otherwise damaged by UV radiation, photosystem efficiency could be compromised (Chen et al. 2019; Lysenko and Varduny 2013). When chloroplasts are shielded from bleaching and damage by high quantities of photoprotective anthocyanin, photosynthesis and the subsequent accumulation of necessary photosynthates becomes more efficient (Chen et al. 2019). Photosynthetic efficiency provides insight on how well a plant is able to generate primary metabolites. Because higher anthocyanin content and thus floral color is correlated with higher photosystem efficiency, it is likely that dark red floral morphology has advantages to growth and reproduction.

Phenotype, including floral morphology, is a combination of environment and genetics (Hoballah et al. 2007). Within and between generations, a plant's phenotype reflects an interplay between its genotype and environmental conditions, with cross-generational interactions being deemed maternal effects. Roach and Wulff (1987) define maternal effects in plants as "the contribution of the maternal parent to progeny phenotype beyond the equal chromosomal contribution of both parents". This contribution is due to the physical connection between the maternal plant and developing offspring via such seed structures as the funiculus. During this

process, the physical attachment between the maternal plant and seed allows the maternal plant to impart hormones directly into embryos after genotype is decided. Maternal genotype can be involved in this process, as it could influence maternal ability to pass on traits through maternal effects. However, maternal effects are also impacted by environmental conditions, as different stressors and stress levels contribute to the hormones present in the maternal plant to be transferred into offspring (Riginos et al. 2007). Maternal effects contribute to evolutionary response to stress in that they provide another way for maternal plants to share beneficial traits and adaptations with their offspring. Because of their proximity in time to seed development, germination and early seedling growth are greatly affected by maternal effects and are thus suitable processes to assess the extent to which maternal effects contribute to plant fitness and viability (Donahue 2009).

Maternal effects can be manifested in plastic chemical responses. Previous studies of abscisic acid expression and stomatal conductance under drought conditions in *Impatiens* have shown that maternal plants experiencing stressful conditions impart maternal effects on the ABA content and sensitivity of their offspring (Maruyama et al. 2016). In the case of *Impatiens*, maternal plants under stress are releasing hormones to tolerate their conditions and transferring these hormones to seedlings via maternal effects. In *Ipomopsis*, maternal plants that are plastically expressing high anthocyanin levels in response to UV stress utilize maternal effects to influence the plastic response of their offspring in order to better prepare them for stressful conditions. In *Ipomopsis* on the front range, anthocyanin content appears to vary in a way that could be either an evolutionary response to high UV levels, or unusually high plasticity in floral color (Milano et al. 2016). In the case of plasticity, both maternal and offspring environmental conditions are relevant to offspring floral morphology. Maternal environment contributes to

adaptations imparted by maternal effects, which influence offspring germination and growth. Simultaneously, seedling environmental conditions also play a critical role in early life history. In this way, if the seedling experiences similar conditions to the maternal plant, maternal effects can strengthen a seedling's ability to tolerate stress by imparting phenotypically advantageous traits to offspring that are specific down to the microclimatic level (Dixon et al. 2001). Variation and variability of anthocyanin content in response to UV stress in *Ipomopsis* could provide insight into the role of maternal effects in evolutionary and life history.

To address the maternal and progeny impacts of UV on fitness, I asked the following experimental questions:

Does maternal flower color affect realized fitness?

- Do seeds from dark and light maternal plants differ in number, biomass, germination rate, or germination success?

What effect does maternal flower color have on the physiology of seedlings?

- Do dark and light maternal plants produce seedlings which differ in photosynthetic potential, biomass, anthocyanin and chlorophyll content?

**Methods**

*Organism*

Scarlet gilia, *Ipomopsis aggregata* (Polemoniaceae) is a biennial-perennial flowering herb native to the western United States. It is characterized by deep scarlet to pink 1.5-5 cm tubular flowers which bloom from a basal rosette from late June through late September (Paige and Whitham 1985). Basal rosettes can overwinter, and bolting occurs after 1-8 years (Paige 1992). The species is self-incompatible with an optimal outcrossing distance of approximately 10 meters (Waser and Price 1989). Pollinators of scarlet gilia include two species of hummingbird (*Selasphorus patycercus* and *rufus)* as well as one species of hawkmoth (*Hyles lineata).* Here, we examine populations of *Ipomopsis aggregata* with subtle flower color variation (dark red to lighter red/pink) at about 8000 feet (2438m) elevation in the Colorado front range of the Rocky Mountains. Variation in flower color has been attributed to plasticity as well as species hybridization.

To explain the wide variety of floral color morphology within *I. aggregata,* some argue that there has been a very recent and shallow speciation event, diverging *aggregata* subspecies *candida* and *collina* due to fitness advantages of dark individuals and pollinator niche differentiation, but the same study postulates the possibility of one species displaying particularly high plasticity of floral color (Milano et al. 2016). It has been suggested that variation in color of scarlet gilia could coincide with different pollinator preferences, with hawkmoths preferring lighter petal shades (Paige & Whitham 1985, Meléndez-Ackerman et al. 1997). However, other exploration into pollinator preference showed little evidence of this correlation, despite lighter variations having a heavier pollen load at night, when the hawkmoths are most active (Elam & Linhart 1988). Previous findings also indicate a particularly high

variability in nectar production and seed number within *I. aggregata* relative to other species of *Ipomopsis,* further supporting theories postulating high levels of reproductive plasticity within the species (Meléndez-Ackerman 1997, Heschel et al. 2007). Consequently, there are high levels of variability in hummingbird pollinator preference in response to plastic nectar, pollen, and color within and among populations (Ornelas and Lara 2015). There is also speculation of hybridization, particularly in the front range, due to contiguous habitats, high levels of gene flow, and flexible pollinator preference between subspecies (Milano et al. 2016, Meléndez-Ackerman 1997; Campbell 2004). Historical findings show reproductive advantages of introducing pollen from large populations of *I. aggregata* into smaller ones despite lack of hybrid vigor, suggesting the existence of an advantageous phenotype which benefits from gene flow (Heschel and Paige 1995).

*Study Site*

Scarlet gilia seeds were collected from 24 dark and light maternal plants form four sites (A, B, C, and D) in Manitou Experimental Forest (MEF). These four sites contain *Ipomopsis aggregata* subspecies collina; plants had relatively shorter corollas and speckled petals at the face of the corolla; also, Colorado College herbarium samples (COCO) only document *Ipomopsis aggregata* and *Ipomopsis aggregata* ssp. collina in this geographic area. MEF is an alpine ecosystem in the foothills of the Rocky Mountains at approximately 8000 feet (2438m). Other common species in the forest include *Pinus ponderosa* and *Populus tremuloides*. The four sites chosen covered a variety of environmental conditions and canopy cover along equally disturbed roadside habitats in the forest. Site A has the least moisture and canopy cover and the highest UV exposure. Sites C and D have moderate amounts of canopy cover, with D having slightly more. Site B has the most canopy cover (lowest UV exposure) and the most soil

moisture. UV exposure measurements were taken with a UV-VIS spectrometer (Ocean Optics) for previous study of identical sites in MEF (Heschel, unpublished data).

*Site Environmental Data Collection*

Soil surface and temperature were recorded on two occasions (June 18th and July 9th) at Manitou Experimental forest (MEF). Measurements were taken using a temperature gun (IR Extech) and temperature probe (Fisher) 1-3cm under the surface of the soil. Three individual readings were taken at each location. There were five locations per site, and five sites: A1, A2, B, C1, and C2. Sites A and C were broken up into two parts because they were large and contained different microhabitats. Temperature readings were recorded as averages of the three readings from each location for both soil and surface temperatures, and then total averages of both the soil and surface temperature readings recorded at each site. The average soil surface temperature taken across sites was 16.48 °C (ranging from 11.5 to 23.8) and the average soil temperature was 15.66 °C (ranging from 13 to 17.5). In order to determine the ideal temperatures for experimental germination, historical May 24th, May 31st and monthly May and June high and low averages were compiled for Woodland Park, Colorado Springs, and Aspen, and considered in combination with soil temperatures taken at MEF. 18 °C was about the average daytime temperature across field data as well as historical weather records from May and June for Woodland Park and Colorado Springs.14 °C was chosen for a low, as compiled data from the surrounding area indicated a maximal difference of approximately 3 °C between day and night. The average amount of sunlight for Woodland Park and Colorado Springs for May and June was found to be 14 hours with intensities ranging from 562 to 1568 $\mu$moles/m$_2$·s across sites. The average UV exposure was 1151.51 $\mu$moles/m$_2$·s. Soil moisture was also measured across sites at MEF on July 9th, 2019 and ranged from 3 to 8 % VWC, averaging 5.13 % VWC.

*Germination Assay*

Grouped by maternal plant, seeds were counted individually using forceps, then washed using deionized water and a sieve, then transferred to petri dishes and placed in the fridge to dry over the weekend (3 days). Plates for agar were irradiated with UV-B and C radiation for 5 minutes to sterilize them. Eight batches of 0.5% agar mixture were prepared with 500 mL tap distilled water and 2.5 g plant cell culture tested agar (Sigma) each. Before the agar mixture was ready, plates were irradiated for an additional 30 minutes, then poured with approximately 15 mL of solution per plate. After the plates were poured, seeds were separated from dried clumps using gentle rolling between fingers, shaken to randomize, and then chosen haphazardly from the center of the dish. Using this sampling method, 8 replicate plates, each with 8 seeds from each maternal plant were placed onto agar plates using identical, fine forceps. For the single plant which did not have enough seeds, additional seeds from a similarly colored maternal plant from the same population were used to bolster seed number. Each plate was then wrapped with parafilm and placed in a dark cold room (5.56-6.67 °C) for approximately four days (plates were placed in black flats & covered in foil). The growth chamber was set to 18 °C during the day and 14 °C at night, with 14 hours of incandescent and LED lights. Plate placement on growth chamber shelves was balanced in order to have equal representation of each maternal plant per shelf, then randomized to determine individual plate placement.

*Censusing*

The day 0 census was taken on June 24th, 2019 before the plates were placed into the chamber. Plates were then censused three times per week (M, W, F) for 21 days. To census, plates were individually removed from the growth chamber and looked at under a dissecting scope, as well as with hand lenses to assess how many of the seeds on the plate were germinated.

On day 0, plates were left in stacks in trays instead of their randomized order and put in the growth chamber due to time constraints, but were placed in their randomized order on day 1. Additionally, on day 0, plates had too much condensation to see through the lids, so parafilm was removed to observe seeds. Plates were then re-wrapped. This was beneficial to encourage gas exchange. If seeds appeared to have grown fungus within the first two censuses, they were removed from the plate, rinsed with deionized water, placed back onto the plate, and these plates were rewrapped with parafilm. Seeds were also separated with forceps if the majority of them had clumped together within the first two censuses. After the first two censuses, plates were left wrapped for as long as possible to discourage contamination. For the final census, plates were unwrapped to ensure a precise final assessment of germination status. On July 16, before physiological data collection began, each plate was assessed for fungal growth and aborted seeds. The number of seeds covered with severe fungal growth was recorded individually for each plate. The number of aborted seeds was removed from the total plate number for statistical analyses.

*Additional Seedling Growth*

After physiological measurements were taken, seeds from maternal plants which had the highest rates of germination were selected to form 11 plates from 16 maternal plants (some maternal plants were combined on plates). 10 seeds were placed on each plate and plates were placed into the growth chamber under identical conditions to the original study in a similar randomized block pattern. After 21 days of growth, physiology measurements were taken in an identical manner on these additional seedlings in order to bolster sample size for analysis of physiological factors.

*Seedling Physiology Measurements*

The six largest seedlings were chosen from each maternal plant for physiological measurements. Each maternal line had six replicates pooled by shelf so as to ensure randomness in choosing seedlings to measure. First, seedlings were placed on a balance (Denver Instruments) in groups of two and their combined mass was recorded.

Photosystem efficiency was measured for each replicate seedling pair using a Plant Photosynthesis Monitoring BV (minippm) EARS fluorometer. The photosystem meter uses a bright light to measure the amount of fluorescence and reflectance off of a leaf in order to estimate the amount of red light the chloroplasts within are absorbing. Two germinated seedlings were lined up within the field of view for the monitor against a matte black background to ensure no extra reflectance was recorded and to cover as much of the field of view as possible without damaging the seedlings. The percent photosystem efficiency for each pair was recorded in fv/fm (variable fluorescence /maximal fluorescence).

The chlorophyll content of each replicate seedling pair was measured using a SPAD meter (CCM 200 plus, Opti-Sciences Inc.). The chlorophyll meter uses two wavelengths of light (one which chlorophyll absorbs, one to account for leaf thickness) to calculate chlorophyll content index (CCI) by measuring the amount of light absorbance by the chlorophyll in the leaf. Each pair of seedlings were placed into the chamber of the meter and the head of the meter was clamped shut tightly on them.

After the chlorophyll content of the seedlings was recorded, their anthocyanin content was measured using an Anthocyanin content meter (ACM 200 plus, Opti-Sciences Inc.). The anthocyanin meter, similar to the SPAD meter, uses one wavelength of light to measure the absorbance of light by the leaf due to anthocyanin to calculate the anthocyanin content index.

This was the last measurement taken on the seedlings, and after they were clamped into the meter's chamber they were discarded.

*Maternal Plant Traits*

During June and July of 2018, physiological measurements were taken on a large portion of the population of dark and light scarlet gilia plants at MEF. Plants were characterized as dark or light by two observers using a Munsell color chart to assess maternal color. An equal number of leaves approximately 20 centimeters off the ground were then selected from dark and light plants to measure photosystem efficiency, anthocyanin and chlorophyll content indices, foliar anthocyanin, stomatal conductance, and leaf temperature. The methodology of measuring photosystem efficiency, chlorophyll, and anthocyanin content on maternal plants in 2018 (as well as 2017 and 2016 for photosystem efficiency), was identical to the seedling physiology methods above, used in 2019.

Stomatal conductance was measured on the lowest fully expanded *Ipomopsis* plant using an SC-1 Porometer (Decagon Devices). Leaves were carefully placed in the same orientation in the cuvette of the device and only measurements between 40 and 600 mmol/$m_2 \cdot s$ were recorded to ensure minimal human error. After stomatal conductance was measured, a different leaf from the one used with the porometer was chosen and an infrared temperature probe (Extech Instruments) was used to measure temperature along the adaxial midrib of the leaf. 14 seeds from both dark and light maternal plants (28 total) were harvested from a similar elevation to MEF in Mueller State Park during the 2016 field season. Seeds were germinated on 0.5% agar and germinated at 20C with 12 hours of light. After germination, seeds were pulverized and dried overnight using a Polytron tissue grinder. Extraction of foliar anthocyanin was performed according to the methods of Dixon et al. (2001), and concentration was calculated using a

measure of absorption of the supernatant at 535 and 650nm (Genesys 20 spectrophotometer, Thermo).

*Statistical Analyses*

All statistical analyses were performed using JMP (version 5.1.2, SAS Institute). Two-way ANOVAs were performed to determine significance of floral (or maternal floral) color and site within MEF on photosystem efficiency, stomatal conductance, anthocyanin content index, chlorophyll content, and biomass. Site from which plants or seeds were harvested was considered a random factor and floral color was considered a fixed factor (mixed model, Type III sums of squares). Anthocyanin content, chlorophyll content, and photosystem efficiency were log transformed and all models were examined with normal probability plots to ensure normality of residuals. The site by floral color interaction was not of interest because site was considered a blocking factor; site was included in analyses to control for micro-site environmental effects.

Linear regression was used to determine strengths of relationships between physiological factors. Every variable was regressed against all other variables and slopes and P values were reported. In order to determine differences in germination success, a Wilcoxon test was used on these non-normal, non-parametric data; germination frequency was calculated on a per plate basis. Kaplan-Meier survival analysis was conducted to determine germination rate differences by floral color. Bar graphs were made using Microsoft Office 2016 (Excel) and show means and standard error information.

**Results**

*Maternal Performance*

Physiological measurements of photosystem efficiency, conductance, anthocyanin content index, and chlorophyll content index taken in June and July of 2018 show significant

effects of floral color on photosystem efficiency, anthocyanin content, chlorophyll content, and marginally stomatal conductance (Table 1, Figure 1). In these cases, plants with darker red floral morphologies have higher photosystem efficiency, stomatal conductance and higher chlorophyll content, as well as higher anthocyanin content, concurrent with their darker color. Additional measurements of photosystem efficiency between dark and light-colored plants also showed significantly higher photosystem efficiency in dark plants in 2017 and 2016 at MEF (Figure 1).

Site had significant effects on conductance, chlorophyll content, and marginally photosystem efficiency (Table 1). Chlorophyll content was similar at sites A and D and slightly higher at B and D. Conductance slightly increased from site A to D. Significant differences in conductance and chlorophyll content between sites are concurrent with levels of UV exposure experienced under different levels of canopy cover at each site. Data collected from previous field seasons shows particularly stressful UV conditions at MEF, likely due to its high elevation (Figure 2). An additional census was done at Rocky Mountain Biological Laboratory (RMBL), which is located at 9500 feet (2896m). At higher elevation, 71 per cent of the total number of *Ipomopsis* plants consisted of dark red colored individuals, whereas at MEF, 61 per cent of the total number of individuals were darkly colored (Figure 3).

| Effect | Photosystem Efficiency | Conductance | Anthocyanin Content | Chlorophyll Content |
|---|---|---|---|---|
| **Floral Color** | 60.5233*** | 3.6852+ | 103.7519*** | 8.2232** |
| **Site** | 2.3353+ | 48.8919*** | 0.0987 | 11.8391*** |

Table 1. F ratios from two-way ANOVA of effects of floral color and site on maternal plant physiological performance. (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, + $p < 0.10$). Maternal plant data was taken in June/July 2018.
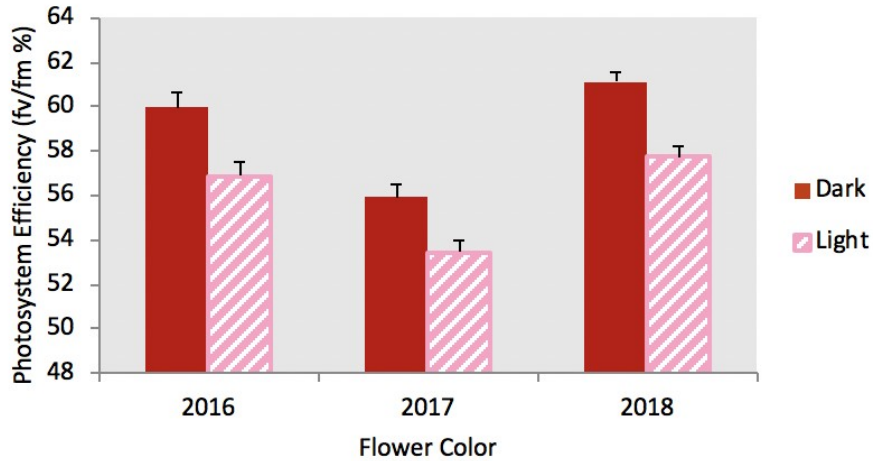
Figure 1. Historical data tracking photosystem efficiency in dark and light maternal plants, as well as two additional summers prior in MEF.
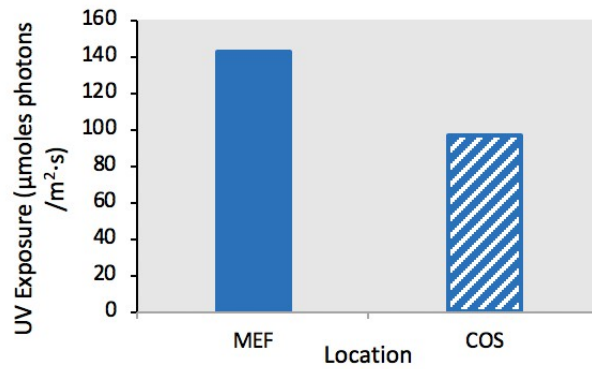


Figure 2. Light intensities at Manitou Experimental Forest (8000ft, 2438m) and Colorado Springs (6000ft, 1829m).
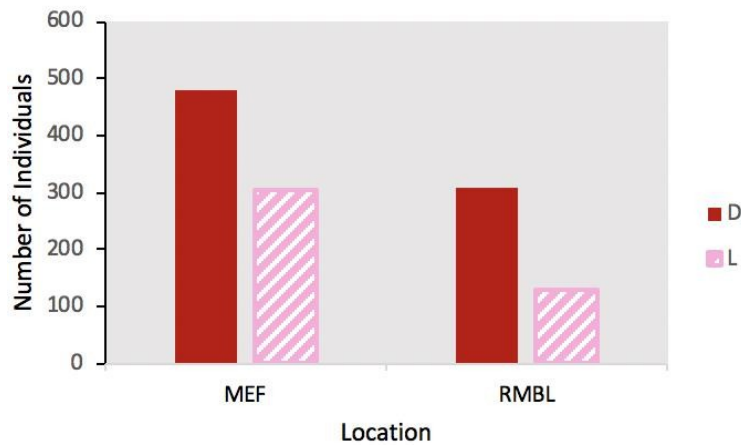


Figure 3. Counts of dark and light plants in Manitou Experimental Forest (MEF) and Rocky Mountain Biological Laboratory (RMBL). MEF is at an elevation of approximately 8000 feet and RMBL is at approximately 9500 (2438m and 2896m). At MEF, light plants represent 39% of the population, whereas at higher elevation at RMBL, light plants represent 29% of the population.

The number of seeds and the average weight of twenty seeds was also taken as an additional measure of maternal performance and fitness. Floral color did not have a significant effect on the number of seeds produced by each maternal plant. Both dark and light maternal plants produced similar numbers of seeds (Figure 4A). Site had a minimal effect on the number of seeds produced (Fig 4B, Table 2). Dark maternal plants did produce significantly more massive seeds than light plants (Fig 4C, Table 2). Site also had a minimal effect on seed biomass, with site B producing slightly more massive seeds than sites A and C not affect seed mass (Figure 4D, Table 3).
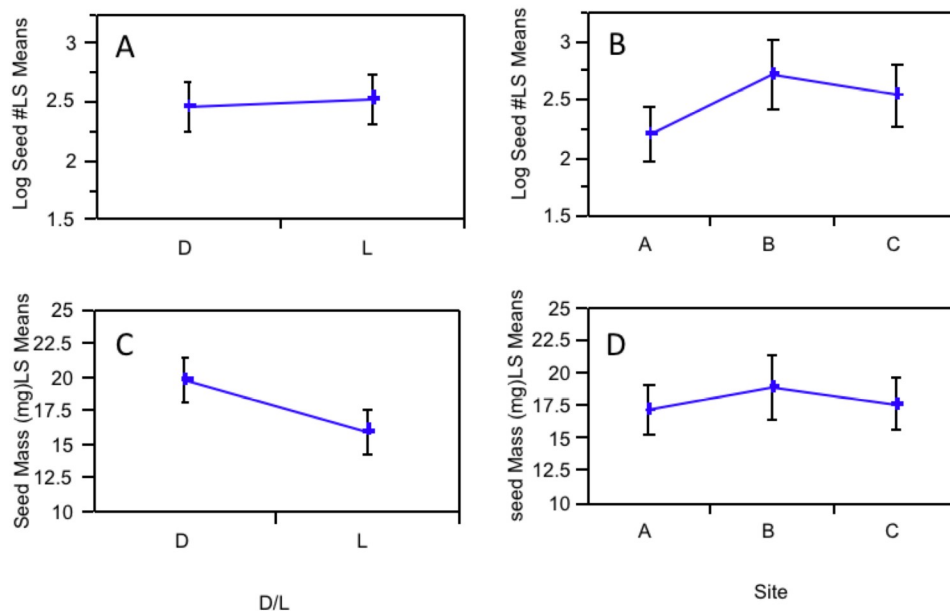


Figure 4. LS means plots of seed number (A and B) and seed mass (C and D) produced by dark (D) and light (L) maternal plants from each site.

*Germination*

There was a significant difference between dark and light maternal plants both in the proportion of their seeds which germinated as well as the germination rate (Figure 5, Table 2). While 49.282% of seedlings from dark maternal plants germinated, only 27.455% of seedlings from light maternal plants had by the end of the 21-day experiment (Figure 5).
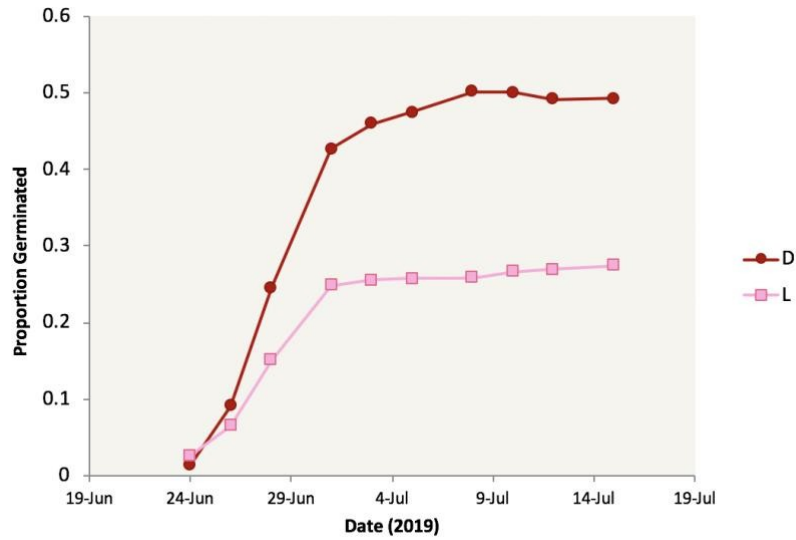
Figure 5. Average germination success of seeds from dark (D) and light (L) maternal plants. Note: 50% germination is typical in this species.
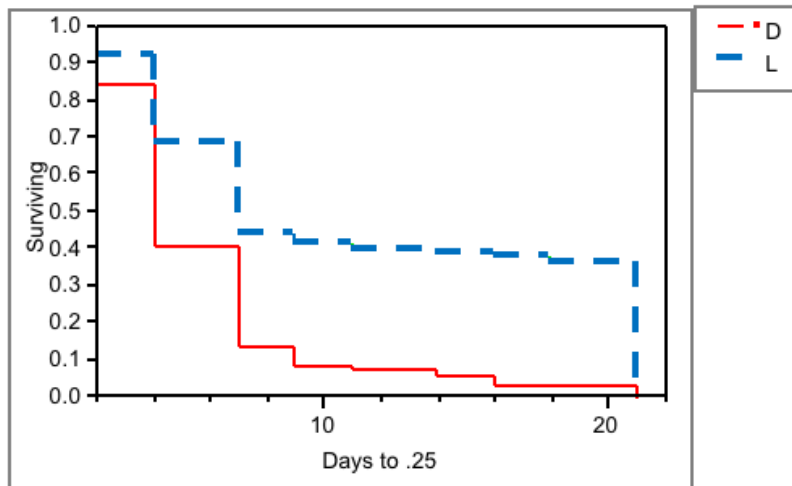


Figure 6. Wilcoxon test survival plot of germination of light and dark seeds.

Germination success after 2, 7, and 14 days were also analyzed to determine differences in germination success between light and dark maternal plants. After 2 days, there was no significant difference in the rate at which seeds from dark and light parents were germinating. However, every time interval after 2 days, as well as the overall rate were significantly different for seeds from light and dark parents (Figure 6). Seedlings from darker maternal plants germinated more

successfully as well as at a greater rate than seedlings from light maternal plants (Table 2, Figures 5 and 6).

| Germination | Chi Square | DF | P Value |
| --- | --- | --- | --- |
| **Success 2 Days** | 3.6148 | 1 | 0.0573 |
| **Success 7 Days** | 34.6813 | 1 | <.0001 |
| **Success 15 Days** | 51.7697 | 1 | <.0001 |
| **Overall Rate** | 25.7935 | 1 | <.0001 |

Table 2. Wilcoxon test of germination success and rate between seedlings from dark and light maternal plants over the course of the 21-day experiment.

*Physiological Factors and Correlations*

Dark and light maternal plants gave rise to seedlings which were significantly different in numerous physiological ways. Seedlings from dark maternal plants had significantly higher biomasses, photosystem efficiencies, as well as anthocyanin content than seedlings from light maternal plants (Figure 7, Table 3). Higher photosystem efficiency in seedlings with dark parents is corroborated by previous data which suggests dark plants growing in MEF have higher photosystem efficiency (Figure 1). There was no significant difference in chlorophyll content between seedlings from dark and light maternal plants (Figure 7, Table 3). Additionally, biomass and chlorophyll content of the seedlings were marginally affected by the site from which they were harvested. Biomass was slightly elevated at site B compared to sites A and C, and chlorophyll content was slightly lower at site C than at sites A and B (Table 3).
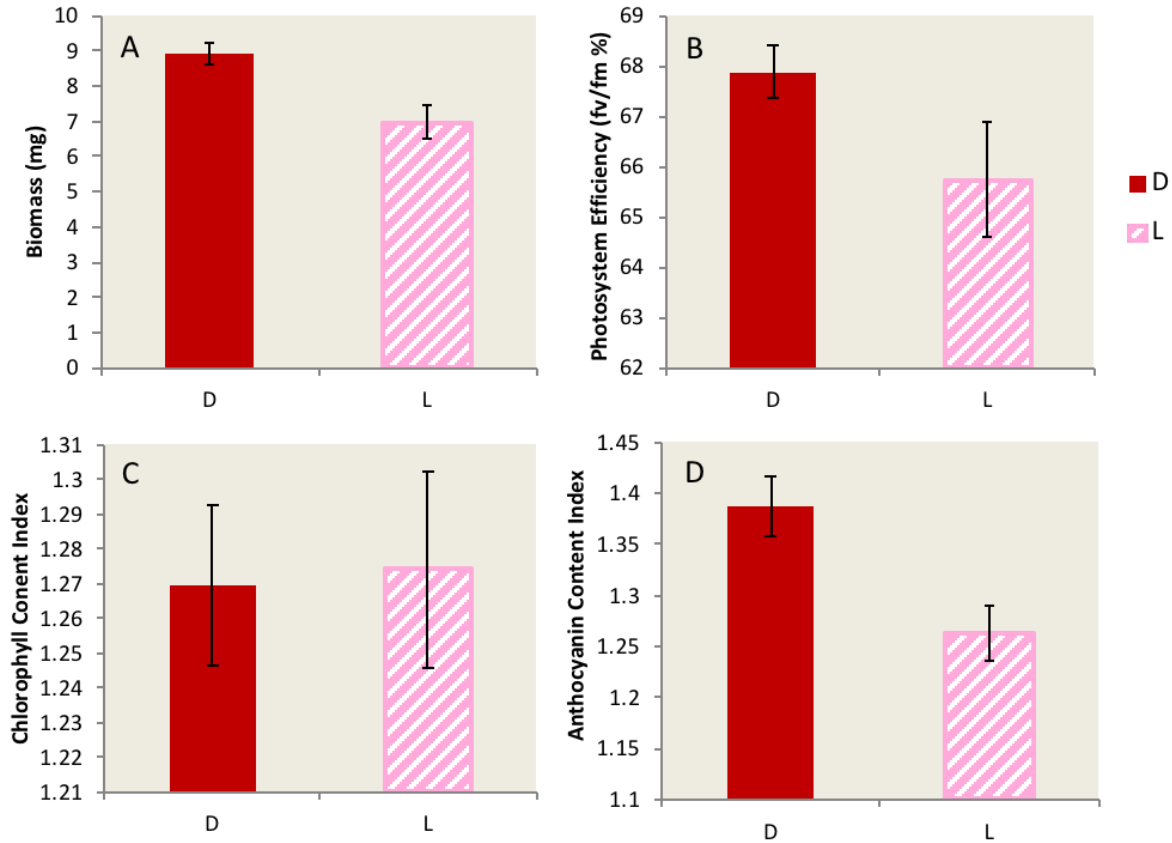
Figure 7. Biomass (A), photosystem efficiency (B), chlorophyll content index (C), and anthocyanin content index (D) of seedlings from dark (D) and light (L) maternal plants. Means ± SE shown

| Effect | Biomass | PE | Chlorophyll Content | Anthocyanin Content |
|---|---|---|---|---|
| **Floral Color** | 9.7752** | 2.9846+ | 0.0002 | 8.3808** |
| **Site** | 3.337* | 0.005 | 3.3758* | 2.2742 |

Table 3. F ratios from two way ANOVA of effects of floral color and site on seedling physiological performance. (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, + $p < 0.10$).

In addition to the relationships between maternal floral color, site, and physiological factors, possible relationships between physiological measurements on the seedlings were also examined. Among physiological factors including average rate of germination for seedlings from each plate, the strongest correlation was found between biomass and photosystem efficiency. Relatively strong correlations were also found between biomass and average germination rate as well as biomass and anthocyanin content.

**Discussion**

*Summary*

UV and anthocyanin levels at MEF are particularly high because it is located at 8000 feet (2438m) (Figures 1 and 2). Under these stressful conditions, maternal floral color (dark & light red) had potential impacts on individual realized fitness as well as throughout the early life history of seedlings. In particular, the impact of dark red morphology on photosynthetic efficiency and anthocyanin content index was shared between maternal plants and their offspring, indicating the likelihood of maternal effects influencing dark and light derived seed viability differently due to differences in stress tolerance. The higher proportion of dark color morphology observed at higher elevations is also consistent with suggested fitness and physiology advantages of high anthocyanin content under UV stress (Figure 3). Overall, higher anthocyanin content and consequent dark morphology seem to provide *Ipomopsis aggregata* with advantages in reproduction and life history, likely due to the molecule's interaction with UV radiation.

*Overall Maternal Effects*

Maternal floral color influenced progeny physiology in numerous ways attributable to maternal effects, genotype by environmental interactions (microclimate), and/or phenotypic plasticity. As was expected, dark maternal plants produced seeds which were more massive than seeds from light maternal plants, however, dark and light maternal plants produced roughly the same number of seeds (Figure 4). Dark derived seed advantage indicates that anthocyanin content could have a role in reproductive fitness through transfer of stress tolerance adaptations between maternal plant and embryo during seed development. Later in the seed's life cycle, a higher proportion of seeds from dark maternal plants germinated at a faster rate (Figure 5 and 6).

Because maternal color seems to influence both seed viability and its sensitivity to suitable germination conditions, anthocyanin may be influencing germination after the dark maternal plant is able to impart its high anthocyanin levels to its progeny.

Physiologically, seedlings from dark red maternal plants had higher biomasses, anthocyanin content, and photosynthetic efficiency than seedlings from light maternal plants (Figure 7). Physiological advantages show directly that be it by chromosomal contribution, environmental conditions, or maternal effects, dark maternal plants are able to impart their anthocyanin concentration to their offspring. Higher anthocyanin content subsequently provides size and photosynthetic advantages even when offspring are not experiencing UV stress. Higher photosynthetic efficiency in seedlings from dark adults is consistent with historical findings that dark adults are able to photosynthesize more efficiently than light, suggesting implications for anthocyanin content and fitness levels across generations of *Ipomopsis aggregata* (Figures 1 and 7B). While the lack of correlation between maternal floral color and seedling chlorophyll content did not agree with significantly high chlorophyll levels in dark adults in previous field seasons, anthocyanin is still likely interacting with chlorophyll in response to UV radiation either under more stressful conditions, or over a more prolonged time scale of exposure (Table 1 and Figure 7C).

*Maternal Effects and Seeds*

While the similarity in number of seeds produced by dark and light maternal plants indicates a lack of potential fitness advantages of dark floral morphology, the higher biomass, germination success, and germination rate of seeds produced by dark red maternal plants imply fitness advantages of high anthocyanin content (Tables 2 and 3). Because *Ipomopsis aggregata* employs a bolting strategy and releases many seeds in a concerted reproductive event, a

maximally efficient number of seeds has likely evolved (USDA plant guide). The basal rosette does not sprout a floral stem until the plant has enough energy stored to maximize fitness in reproduction, so it is likely that plants produce approximately the same number of seeds regardless of color morphology. However, because light individuals have experienced more stress due to lack of UV protection, they may take longer to produce seeds. Lower photosynthetic efficiency in light adult plants during previous field seasons could also impact the amount of time a light adult would take to accumulate the necessary nutrients to bolt (Heschel, unpublished data, Figure 1). While there was no difference in the number of seeds produced by dark and light maternal plants, seeds from dark individuals were significantly more massive (Figure 4C). Seed size is correlated with the amount of nutrient support the seed contains to support the plant embryo and early seedling, as well as with maternal effects (Roach and Wulff 1987). Thus, dark maternal plants have a fitness advantage in that their embryos and seedlings have access to better nutrient support and should be more likely to germinate. Interestingly, previous studies of *I. aggregata* relative to *I. tenuituba* show significantly higher and more variable seed production in *I. aggregata*, suggesting possible plasticity in seed production that was not visible in the MEF population, and further advantages of anthocyanin (Meléndez-Ackerman and Campbell 1998).

A higher proportion of seeds from dark red maternal plants germinated at a higher rate, further delineating the fitness advantage of adults with higher anthocyanin content (Figure 5 & 6 and Table 2). High germination success is likely a result of higher quality endosperm of dark individuals and has been used as evidence of maternal effects in previous research (Roach and Wulff 1987). Previous studies have found that during buckwheat seed development, anthocyanin was in particularly high concentrations during the development of the endosperm (Song 2016).

Because high anthocyanin content is correlated with high photosystem efficiency, this finding suggests that darker individuals are able to accumulate more photosynthate to incorporate into seedling nutritional support. Seeds from dark maternal plants having more nutritive endosperm is supported by their higher seed weight and germination success, suggesting they "inherit" both high anthocyanin content and high quality photosynthate via maternal effects. Thus, dark individuals are able to provide better support for their seeds, increasing their relative fitness by genes, maternal effects, or a combination of the two. Additionally, higher anthocyanin content has been correlated with higher levels of expression of biosynthesis genes (Ahmed 2009). Because faster rates of biosynthesis would allow for faster growth, higher anthocyanin concentration in maternal plants and their seeds could be responsible for their faster rates of germination, as well as higher biomass in seedlings.

*Maternal Effects and Seedlings*

Maternal effects are highly visible during germination, and the most visible during the seedling stage (Roach and Wulff 1987, Riginos et al. 2007). Here, maternal effects influenced seedling anthocyanin, biomass, and photosystem efficiency in ways indicating advantages of the dark red maternal phenotype (Figure 7A, B, and D). The larger biomass of seeds from dark maternal plants translates to larger biomass of dark seedlings later in their life history. This could be evidence of nourishment provided by higher quality endosperm catalyzing other life-long advantages, including size. The larger biomass of seeds from dark maternal plants translates to larger biomass of dark seedlings later in their life history (Figures 4C and 7A). Larger biomass could thus be evidence of nourishment provided by higher quality endosperm stimulating other life-long advantages. Anthocyanin content index in seedlings was concurrent with their maternal plant anthocyanin content index, indicating that seedlings from dark maternal plants "inherited"

parental coloration (Table 1 and Figure 7D). Continuity in coloration between generations is important in that fitness advantages of dark color morphology are passed between generations and are visible very early in dark derived progeny. Advantages in stress protection cannot be implemented too early, as cotyledons emerge and begin to photosynthesize soon after germination. Significantly higher photosystem efficiency in dark derived seedlings conveys the "heritable" advantages of anthocyanin (Figure 7B). In past field seasons, higher photosystem efficiency of maternal plants in conjunction with larger seeds, more effective germination, and later higher photosystem efficiency of seedlings indicates that anthocyanin can benefit multiple parts of the plant's life cycle, particularly early seedling life history. These benefits to seedlings are likely due to UV stress relief in adult individuals allowing for higher productivity, subsequently imparting quality photosynthate and high anthocyanin content to seedlings.

The lack of influence maternal floral color had on seedling chlorophyll content also provides insights on the advantages of anthocyanin in *Ipomopsis* (Figure 7C). Because historical data shows darker adult plants have significantly higher chlorophyll content, anthocyanin could be having prolonged effects on chlorophyll that are not visible in seedlings (Table 1). Adult maternal plants had been living under stressful UV conditions for multiple years, while although exposed to UV during development in the field, seedlings had been experiencing no UV radiation in the growth chamber. Thus, protection from UV damage by higher anthocyanin content could be preventing chlorophyll degradation over time in dark adults over the multiple years plants can exist as rosettes before bolting. This advantage of higher anthocyanin content would not be visible in seedlings, as they have experienced significantly less UV stress than adults. Similarity in chlorophyll content between dark and light derived seedlings could also suggest that chlorophyll content is not passed from maternal plant to seeds through maternal

effects to the same extent that anthocyanin content is, perhaps because it is not as variable in response to stress between light and dark adults, or because the effects of anthocyanin on chlorophyll build very gradually in maternal plants.

*Population Distributions of Flower Color*

The correlation of higher anthocyanin content with such numerous possible fitness and physiological advantages brings to question the persistence of light floral color morphology under the stressful conditions of the Rocky Mountains at MEF and RMBL (Figure 3). One previously postulated theory suggests the divergence of color morphology due to pollinator availability and preference of hummingbirds and hawkmoths (Paige and Whitham 1985). If this were true, dark and light floral distribution should correlate with their respective pollinator distribution. However, the higher prevalence of dark individuals at higher elevations is not concurrent with pollinator distribution, as hummingbirds cannot persist at elevations as high as hawkmoths due to low temperatures (Welch and Suarez 2008). One possible explanation for this disjointedness between pollinator and morphology distribution is that although dark individuals are more stress tolerant than light individuals, the larger range of pollinators which prefer light petal color allows the light morphology to persist despite fitness disadvantages. Additionally, lightly colored individuals have been shown to display higher pollen load at night (Paige and Whitham 1985). Different timing of pollen load could contribute to divergence in pollinator preference between light and dark morphologies, as well as afford light individuals more opportunities for pollination by a wider range of pollinators by decreasing competition with dark individuals for hummingbirds.

Light floral morphology may also persist in the front range due to differences in seasonal timing of pollinator availability and reproductive life history. Because hawkmoth migration

through the Rocky Mountains happens later in the season than hummingbird migration, light individuals could be on a different reproductive schedule than dark (Paige and Whitham 1985). If lightly colored adult plants are pollinated later than darkly colored adults, they would develop and release seeds later in the season. Decreased competition for conditions suitable for germination between seeds from dark and light maternal plants could allow light seeds to germinate and grow successfully despite disadvantages in size and likely endosperm quality. As suggested above, the higher photosystem efficiency of dark adult plants likely allows them to reproduce earlier than light adults because of their more efficient photosynthate accumulation. Different seasonal pollinator availability combined with the possible difference in timing of reproduction between light and dark maternal plants likely significantly affects competition between light and dark seedlings, allowing light floral morphology to persist despite fitness and stress tolerance drawbacks.

*Conclusions*

There is strong evidence that maternal effects in *Ipomopsis aggregata* are providing progeny of maternal plants with dark floral morphology with physiological and life history advantages. Life history advantages of dark derived progeny imply increased realized fitness of adult individuals with dark petal color. Cross-generational transmission of anthocyanin content alongside larger seeds and seedlings, higher germination success and rate, and more efficient photosynthesis in dark derived seeds and seedlings indicates that maternal effects are important in *Ipomopsis,* significantly influencing life history and stress tolerance. Further understanding the role of maternal effects on anthocyanin in the fitness and physiology of *Ipomopsis* along the elevation and UV stress gradient of the Rocky Mountain front range could provide insights on the unusual distribution of floral color across these ecosystems.

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# Appendix

| Variable 1 | Variable 2 | Slope | P Value |
|------------|------------|-------|---------|
| **Biomass** | **ACI** | 13.084427 | 0.0134 |
| **Biomass** | **CCI** | 4.9090956 | 0.4198 |
| **Biomass** | **PE** | 26.63523 | 0.003 |
| **Biomass** | **Germination Success** | 5.9788693 | 0.0043 |
| **PE** | **ACI** | 0.006546 | 0.9262 |
| **PE** | **CCI** | 0.0547312 | 0.4937 |
| **PE** | **Germination Success** | 0.002379 | 0.933 |
| **CCI** | **ACI** | 0.0729176 | 0.6176 |
| **CCI** | **Germination Success** | 0.1157088 | 0.025 |
| **ACI** | **Germination Success** | 0.0104238 | 0.8086 |

Table I. Pairwise correlations of all possible combinations of physiological factors, including average germination by plate after 21 days. Slope and P value of linear regressions are shown.

| | Air (Night/Day) (°C) | Soil (°C) | Soil Surface (°C) | Soil Moisture (VWC) | UV Exposure ($\mu$moles/m$_2 \cdot$s) |
|--|--|--|--|--|--|
| Range | | 13-17.5 | 11.5-23.8 | 3.0-8.0 | 562-1568 |
| Average | 14/18 | 15.66 | 16.48 | 5.13 | 1151.51 |

Table II. Averages and ranges in environmental conditions in MEF and surrounding areas (Woodland Park, Aspen).