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The Importance of Outcrossing of Highbush Blueberry Cultivars Blueray and Bluecrop

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ABSTRACT

Some flowering plants require cross pollination, while other rely on selfing. Many, like highbush blueberries, use both, but one method may still be more effective. Cross pollination has been studied in blueberry cultivars which have shown a variety of results. I looked at if outcrossing improved berry traits in cultivars Blueray and Bluecrop. I pollinated Blueray and Bluecrop flowers with within-bush, within-cultivar, and between-cultivar pollen. The proportion of flowers that set fruit, berry mass, viable seed count, proportion of viable seeds, large seed count, proportion of large seed count, and sugar content of berries were compared across cultivars and pollination treatment. Fruit set, viable seed count, proportion of viable seeds, and sugar content showed no significant difference. However, for both cultivars, within-cultivar crossing resulted in smaller berries than selfing or between-cultivar crossing. Additionally, large seed count and proportion were significantly greater in cultivar-crossed berries. Upon this finding, I looked at the correlation between large seeds and mass and found significance. Although bush-crossing negatively impacted the blueberries, cultivar-crossing improved them, so outcrossing between Blueray and Bluecrop produces mixed results. Overall, outcrossing did not have a net positive effect on Blueray and Bluecrop blueberries and potentially should be avoided by farmers.

INTRODUCTION

The reproductive success of flowering plants depends on both donating and receiving pollen. The pollen of some plants is dispersed by abiotic sources such as wind and water, while others depend on animals like birds or insects to move pollen within and among flowers. More than 80% of all flowering plants depend on animals for pollination (Ollerton et al. 2011). In foraging, pollinators often move from flower to flower on the same plant until they encounter several unrewarding flowers and give up and fly off to another plant. Such behavior results in the transfer of both pollen from another plant that they arrived with (outcross pollen), as well as the transfer of pollen from the same plant (self-pollen); (Thomson & Plowright 1980).

Although obligate self-pollination and cross-pollination both occur (Miller & Owens 2009), about half of all animal-pollinated species of plants have a mixed mating strategy in which they use both self and outcrossed pollen (Vogler & Kalisz 2001). Each of the individual mating strategies may provide benefits for the plant. Self-pollination is beneficial if there are limited or inefficient pollinators as pollinators are more likely to visit the flowers nearby, so there is less loss of pollen (Roger et al. 2013). Gervasi and Schiestl (2017) explored ways pollinators may have influenced the evolution of *Brassica rapa's* mating system. Insufficient pollination resulted in morphological changes that enhanced selfing. Specifically, *Brassica rapa* produced smaller, more tightly closed flowers, with reduced nectar and scent, and male and female organs closer together. While the olfactory traits entice pollinators to stay at the same plant where the scent was more apparent, the shape and layout of the flower make it easier to transfer pollen within the flower (Gervasi & Schiestl 2017). However, self-pollination can also be costly. Prolonged self-pollination for generations can lead to inbreeding depression due to the high chances of harmful recessive alleles being inherited from both parents (Hossaert-McKey & Bronstein 2001). Although harmful alleles may be present at low levels in the population, homozygosis allows their expression, which can lower the plants' fitness (Charlesworth 2009). For example, a common fatal abnormality is chlorophyll-deficient albino seedlings, most notably in *Mimulus guttatus* (Willis 1992) and, in blueberries at zygotic levels, this can take the form of misshapen (shrunken or flat) seeds leading to embryonic failure, reducing fertility, and sometimes leading to sterility (Krebs & Hancock 1988). Cross-pollination allows for greater genetic diversity, which can help prevent these issues (Charmantier et al. 2014).

Highbush blueberry, *Vaccinium corymbosum* (Ericaceae), is an important specialty crop in Vermont and a valuable horticultural species in New England. It has a mixed-mating system, in which plants can use both self and outcross pollen (Bieniasz 2007). It is "buzz pollinated," requiring the vibration of bees' thoracic muscles to release pollen while they collect floral rewards (nectar and pollen) (Buchmann 1985). In particular, *Bombus* (bumblebees), *Megachile* (leafcutter bees), and *Osmia* (mason bees) pollinate blueberry flowers (Nooton 2020, Sabine et al. 2020). As optimal foragers, bees will remain at a flowering bush until they find several unrewarding flowers, making them excellent pollinators for both self and cross-fertilization (Waddington & Holden 1979). Although most blueberry cultivars can use their own pollen for fertilization (MacKenzie 1997), which may fall passively on the stigma while the bees forage or may be moved by bees to other flowers within the same plant, others are more productive with cross-pollination. Berry production can also be dependent on what cultivar the outcrossed pollen is from (Mackenzie 1997). However, studies of outcrossing rates in highbush blueberries, even within the same cultivar, have yielded inconsistent results. For example, Bluecrop benefits from self-pollen, as cross pollination lowers fruit set (Mackenzie 1997, Bieniasz 2007), but the opposite was shown in Ehlenfeldt (2001). Some studies have even shown no effect at all (Dogterom et al. 2000).

While Bluecrop has had extensive pollination research, showing a variety of results, Blueray has little to no research on the degree to which Blueray uses self- versus out-cross pollen. Additionally, Blueray has not been crossed with Bluecrop for research. I undertook a hand-pollination experiment to address whether reproduction in Bluecrop and Blueray cultivars differed with self versus outcross pollen. Specifically, I asked: if Bluecrop and Blueray are pollinated with selfed and outcrossed (within and between cultivars) how would it affect their reproductive traits?

METHODOLOGY

Study Site

This study took place on Newbury Blueberry Farm (**Figure 1**), located in Newbury, Vermont, 44.1283° N, 72.1342° W. The bushes at Newbury Blueberry Farm were 18 years old in 2021, having been acquired as saplings aged 18-24 months. Two rows of each cultivar, Bluecrop, and Blueray, alternate for eight total rows, each about 50 plants long. The owners reported that they have a diverse community of natural pollinators (**Figure 2**). Blueray and Bluecrop at this farm flower at the same time from May 23, 2022, to June 6, 2022.


Figure 1: Newbury Blueberry Farm, October 5, 2020


Figure 2: Bumble bee pollinating Bluecrop flowers at Newbury Blueberry

To examine the importance of cross-pollination, I conducted hand pollination experiments for the two cultivars. Branches of plants in both cultivars were pollinated with their own pollen (within-bush crossing), the pollen of other plants with the same cultivar (within-cultivar crossing), or the pollen of other plants in a different cultivar (between-cultivar crossing). To do so, I used 10 bushes of Bluecrop and 11 bushes of Blueray. All plants selected were not on the edge of the field to avoid confounding variables but were otherwise picked at random. Each bush had three bagged branches for treatments and an extra two for pollen collection. Bags were made of bridal veil fabric and about 18 inches long to cover approximately 20-40 inflorescences per bag (**Figure 3**). All of the flowers within the bagged branches were assigned to one of three treatments: 1) flowers were self-pollinated with pollen from the same bush, 2) flowers were pollinated with pollen from another bush of the same cultivar, and 3) flowers were pollinated with pollen from the other cultivar, resulting in six treatment types as follows: Bluecrop flowers with pollen from the same bush, Bluecrop flowers with outcrossed Bluecrop pollen, Bluecrop flowers with outcrossed Blueray pollen, Blueray flowers with pollen from the same bush, Blueray flowers with outcrossed Blueray pollen, and Blueray flowers with outcrossed Bluecrop pollen. The bags prevented natural pollinators from visiting flowers and thus ensured that the only pollen the flower received was that delivered by hand.


Figure 3: Bridal veil fabric bag set up. Tag indicates bush and pollen type

To pollinate flowers, I collected pollen from branches bagged using a Vegibee™ hand-held sonicator to release the pollen into a petri dish (**Figure 4**). This method replicates how bees pollinate in the wild. When they enter a flower, the vibrations from their thoracic muscles used

Figure 4: Hand pollination. Left photo - collection of Blueray pollen using Vegibee. Right photo - painting of Bluecrop pollen onto stamen of floret

for flying (sonication) shake the pollen loose from the anthers (King 2003). From there it falls and sticks to their body. The bee, having collected the nectar in a flower, moves on to another, where the pollen is transferred onto the sticky stigma (Hoffman et al. 2018). To mimic the bees, I transferred this pollen to the bagged flowers using an artist's paintbrush (**Figure 4**). I used a clean petri dish and paintbrush for each transfer and the longest time between collection and pollination was at most 2 hours.

I completed each cycle of hand pollination (all three treatments complete) over a two-day period. Depending on the weather, there were two or three days between the start of each pollination cycle to ensure all flowers were pollinated throughout the flowering season. During the two-week flowering period, the full cycle was completed at least four times. On May 28th, I counted the number of flowers in every bag and later used this to examine the proportion of flowers that produced fruits (i.e., berries). Berries began ripening fully in mid-July and the number of berries in each bag was recorded (**Figure 5**). On July 18, 2022, the collection of samples began. I collected up to five fully ripe berries at random per bag each day. The random selection of berries was completed roughly every three days until a bag had 15 berries collected from it. This took until September 3rd when the last of the ripe berries were collected.

Figure 5: Blueberries on July 19, 2022 at various ripe stages

Of the 15 berries collected from each plant, I randomly selected 10 for further analysis. I weighed each (to the nearest 0.01g), and recorded berry mass, seed count, and sugar content. Seeds were divided into three categories, small (unfertilized), medium, and large (fertilized) (**Figure 6**). I measured sugar content by obtaining juice from each berry which was then placed on a refractometer and the BRIX index was recorded. BRIX is a measure of the percent of sugar dissolved in a liquid.

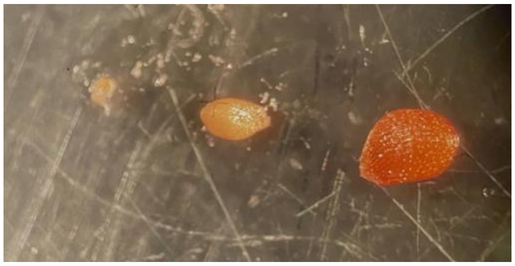

Figure 6: Seed categories, small, medium, large respectively

Data Analysis

I analyzed whether each flower would fruit (yes/no) using a bimodal model after transforming the data logarithmically. I analyzed mass and sugar content with four different models: linear, linear with Poisson family function, and the same models with interaction included. The residuals from all four models were examined to ensure normal distribution, then I ran Akaike information criterion (AIC) tests to determine which of the three models best fit the data. No significant interactions were detected, and the linear regression model was the best fit model for both mass and sugar content.

To examine the effects of hand-pollination treatment and cultivar on the proportion of viable seeds produced per berry, I used a two-way analysis of variance after arcsine transforming the data to improve normality. I analyzed the proportion of large seeds produced per berry in the same way. Viable seed count, which was not normally distributed, was analyzed with two models: a linear model with a Poisson family function and a non-binomial linear model with a Poisson family function. After an AIC test, it was determined that the latter was a better fit for both. Lastly, I analyzed large seed count with three different models: linear, linear with a Poisson family function, and non-binomial linear model with a Poisson family function. AIC tests showed that the former was a better fit.

All models also accounted for the repeated measures design (three treatments/plant) by using "Plant ID" as a random, predictor variable. Finally, I ran EMMs (estimated marginal means) post hoc tests for multiple comparisons of every model.

RESULTS

Overall, an average of 83.46% of flowers produced blueberries after treatment. However, the proportion of flowers that became fruit was not significantly different between pollination treatment ($F = 0.25_{2, 63.1}$, $P = 0.72$, **Figure 7**) or cultivars ($F = 0.54_{1, 64.5}$, $P = 0.72$, **Figure 7**), and there was no significant interaction between the two ($F = 0.01_{2, 67.5}$, $P = 0.25$, **Figure 7**).

Berry mass did not differ between cultivars ($F_{1, 61.2} = 0.35$, $P = 0.56$, **Figure 8**) but significantly varied by pollination type ($F_{2, 68.5} = 5.42$, $P = 0.00653$, **Figure 8**). Hand pollination among bushes within the same cultivar produced significantly smaller berries than selfing ($F = 5.42_{1, 102}$, $P = 0.0056$, **Figure 8**) and cultivar-crossing ($F = 5.42_{1, 64}$, $P = 0.09$, **Figure 8**) with an average of 1.45g while selfing and cultivar-crossing had similar averages, 1.56g, and 1.56g respectively ($P = 0.75$, **Figure 8**).

Viable seed counts were also not significantly different between cultivars ($F = 0.56_{1, 69.7}$, $P = 0.99, 0.45, 0.50$, **Figure 9**) or pollination treatment ($F = 0.86_{2, 63.8}$, $P = 0.47$, **Figure 9**) and there was no significant interaction between the cultivar and pollination treatment ($F = 0.35_{2, 67.9}$, $P = 0.35$, **Figure 9**).

Furthermore, the proportion of total seeds that were fertilized and viable did not significantly vary between cultivars ($F_{1, 62.5} = 0.09$, $P = 0.77$, **Figure 10**), pollination treatment ($F_{2, 67.2} = 2.23$, $P = 0.12$, **Figure 10**), and there was no significant interaction between the two ($F_{2, 64.8} = 1.43$, $P = 0.25$, **Figure 10**).

However, on average, berries from between-cultivar crosses produced 10 more (± 3) large seeds than those in the other treatments ($F_{2, 72.5} = 0.09$, $P = 0.001$, **Figure 11).** Outcrossing between cultivars also resulted in a greater proportion of large seeds per berry. Between cultivar crosses produced 43% (± 1%) large seeds, versus 31% (± 1%) for those within cultivars and 30 % (± 1%) for those crosses within the same bush ($F_{2, 68.2} = 8.57$, $P < 0.0003$; **Figure 12**). Additionally, the proportion of large seeds/berry did not differ between the two cultivars ($F_{1, 62.1} = 0.05$, $P = 0.82$, **Figure 12**) and there was no significant interaction between cultivar and pollination type was not significant ($F_{2, 65.4} = 2.26$, $P = 0.11$, **Figure 12**).

I also looked at the correlation between large seeds and berry mass. Large seed count accounted for 12.56% of the variation in berry mass ($F_{1, 577} = 84$, $P < 2.2e - 16$, **Figure 13**), and large seed proportion accounted for 15.16% ($F_{1, 577} = 104.3$, $P < 2.2e - 16$, **Figure 14**). Large seed proportion and large seed count are not independent so the variation in mass they each cause is also not independent.

Last, on average, the blueberries had 12.38 grams +/- 0.12 standard error of sucrose per 100-gram solution. Although selfing tended to produce berries with 0.7% more dissolved sugar, berry sugar content did not vary significantly between cultivars ($F_{1, 63.5} = 2.83$, $P = 0.10$, **Figure 15**) or pollination type ($F_{2, 73.3} = 1.15$, $P = 0.32$, **Figure 15**), nor was there an interaction between the two ($F_{2, 70.1} = 0.32$, $P = 0.73$, **Figure 15**).

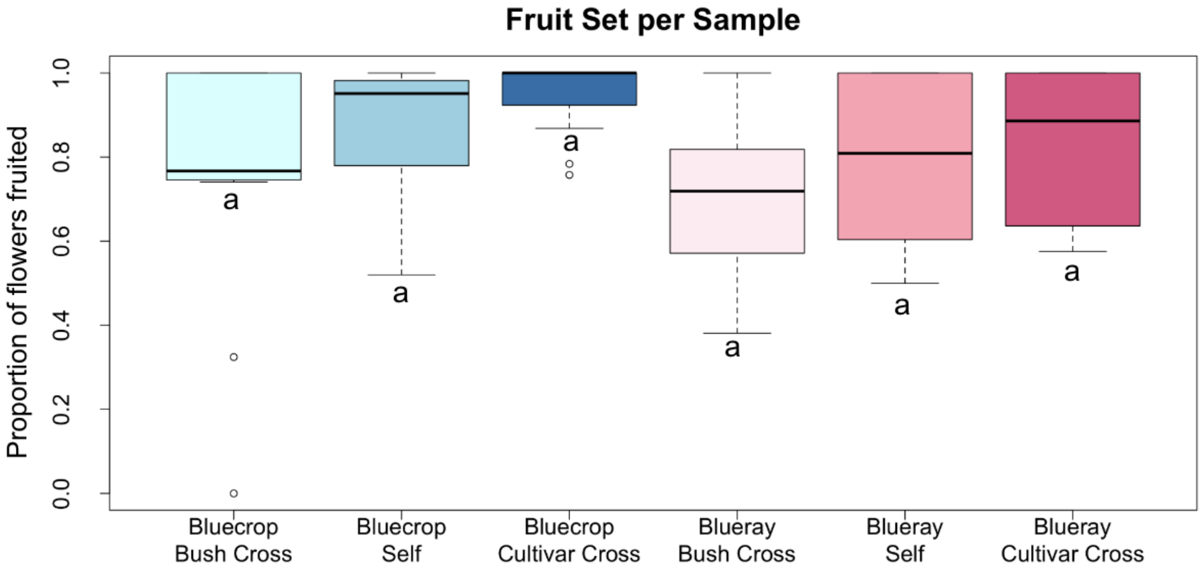

Figure 7: Proportion of flowers that became blueberries per sample in each treatment group. (Untransformed data) Blue = Bluecrop, Pink = Blueray, Light = pollination from a different bush of the same cultivar, Medium = pollination from the same bush, Dark = pollination from a different cultivar (P = 0.7234)

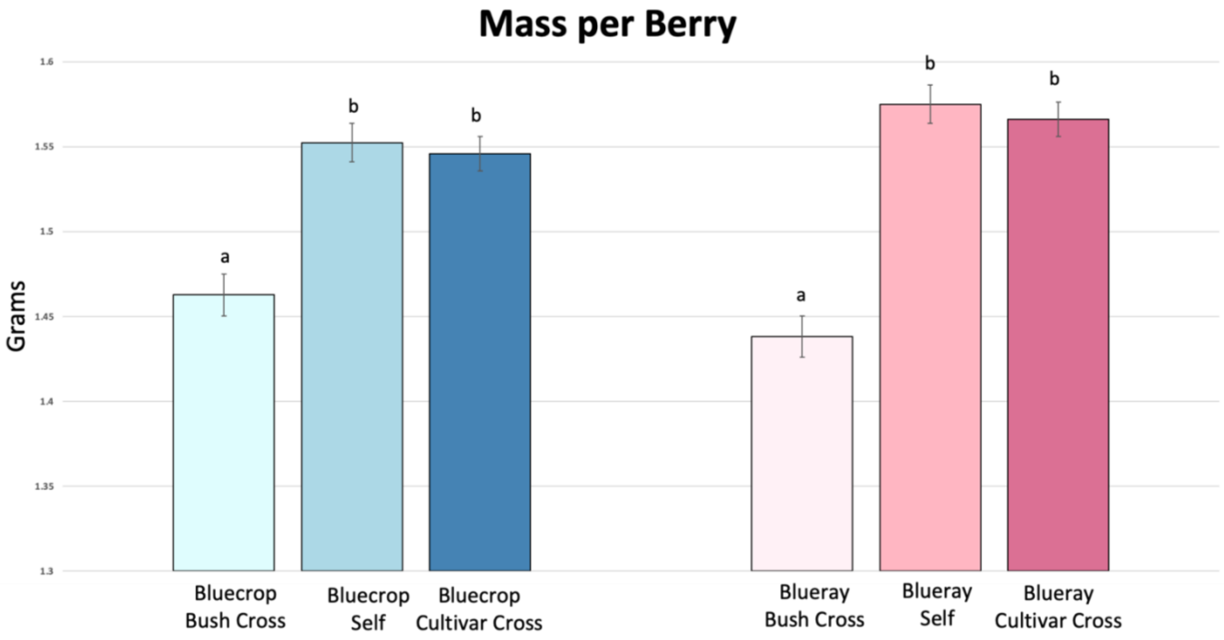

Figure 8: Mass in grams per berry in each treatment group. Blue = Bluecrop, Pink = Blueray, Light = pollination from a different bush of the same cultivar, Medium = pollination from the same bush, Dark = pollination from another cultivar (P = 0.00653)

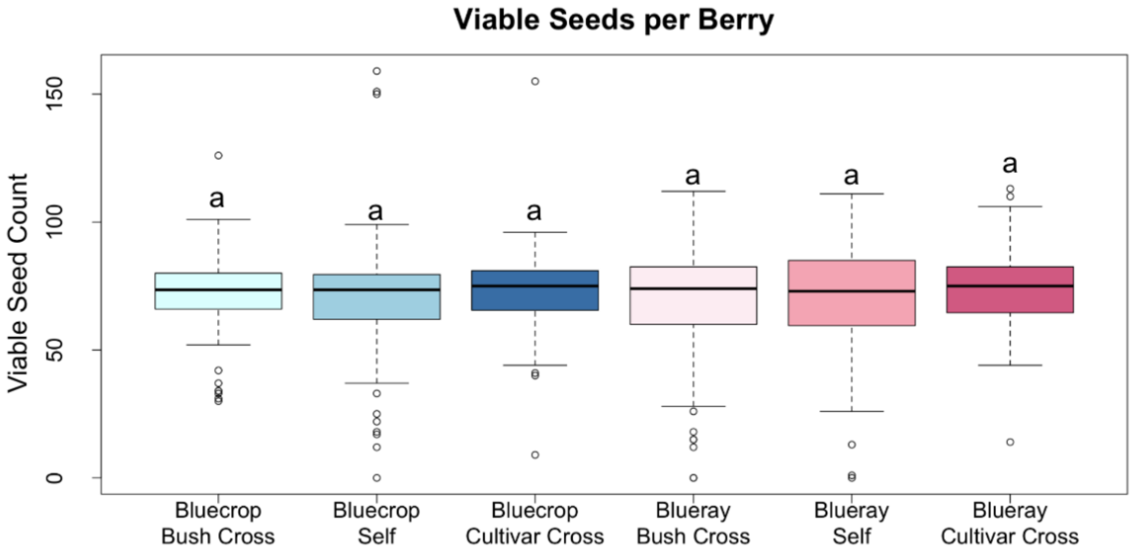

Figure 9: Number of large and medium seeds from each berry within each treatment group. Blue = Bluecrop, Pink = Blueray, Light = pollination from a different bush of the same cultivar, Medium = pollination from the same bush, Dark = pollination from a different cultivar (P = 0.4707)

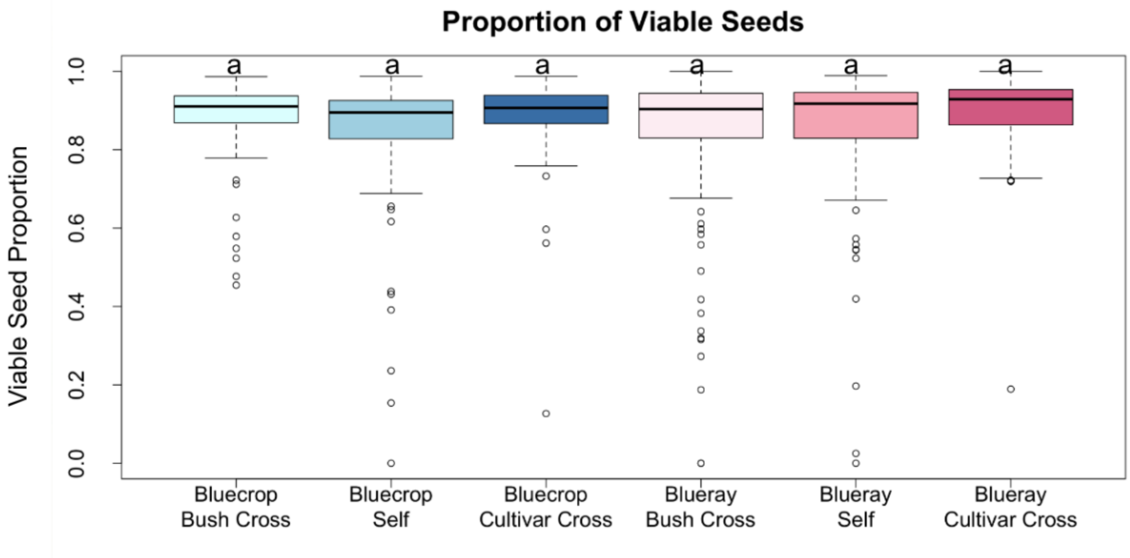

Figure 10: Proportion seeds that were fertilized per berry within each treatment group. (Untransformed data) Blue = Bluecrop, Pink = Blueray, Light = pollination from a different bush of the same cultivar, Medium = pollination from the same bush, Dark = pollination from a different cultivar (P = 0.1150)

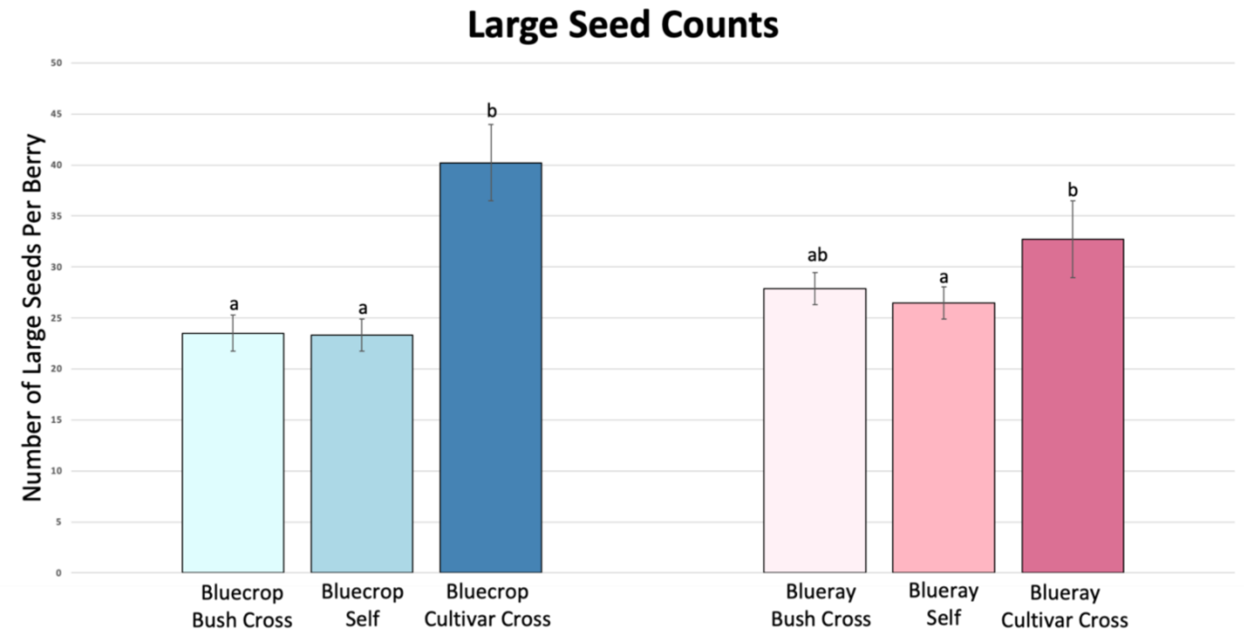

Figure 11: Number of large seeds from each berry within each treatment group. Blue = Bluecrop, Pink = Blueray, Light = pollination from a different bush of the same cultivar, Medium = pollination from the same bush, Dark = pollination from a different cultivar (P = 0.0009401)

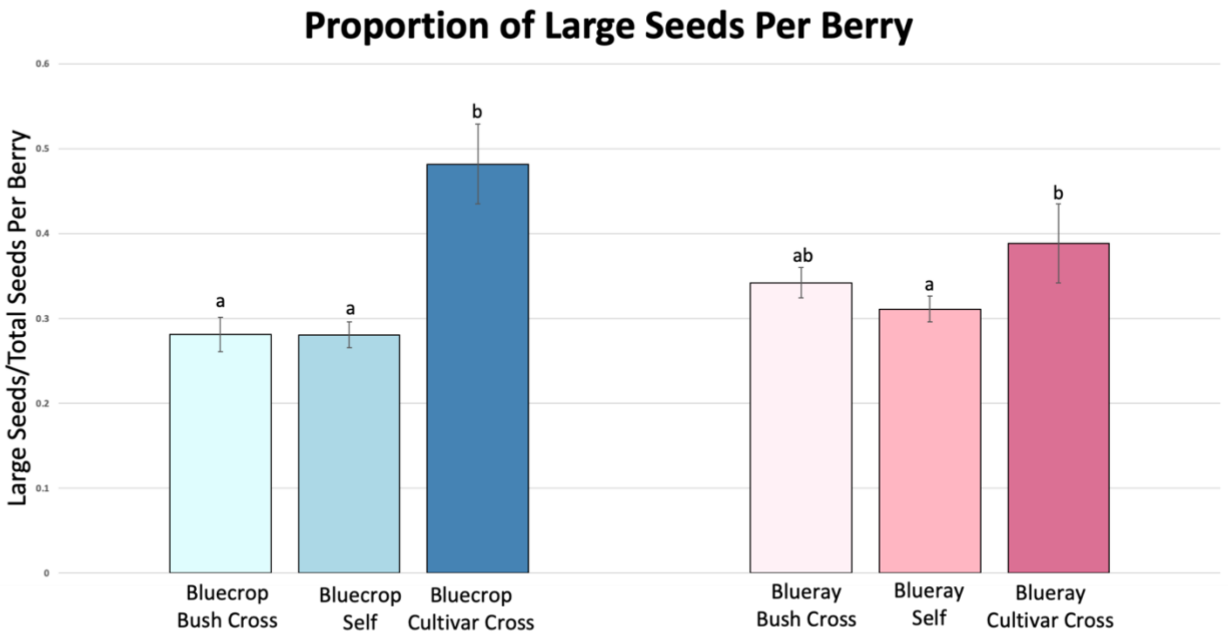

Figure 12: Proportion of large per berry within each treatment group. (Untransformed data) Blue = Bluecrop, Pink = Blueray, Light = pollination from a different bush of the same cultivar, Medium = pollination from the same bush, Dark = pollination from a different cultivar (P = 0.0004801)

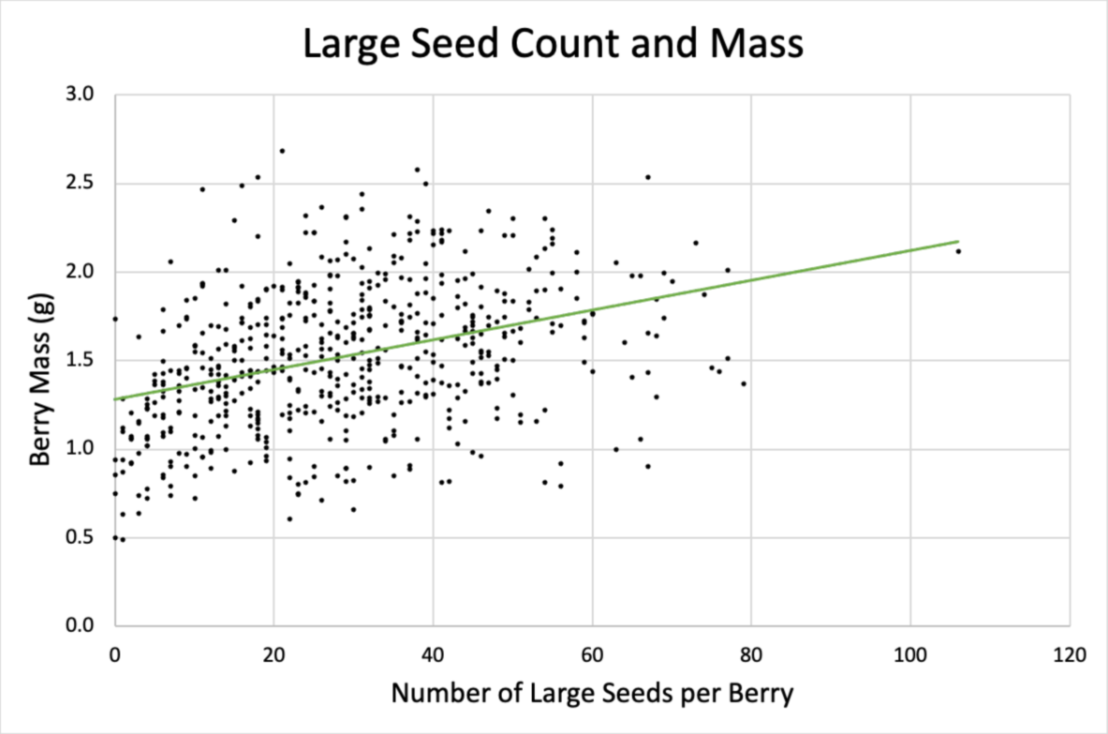

Figure 13: Correlation of the number of large seeds per berry to its mass. Green line = line of best fit. $R^2 = 0.1516$ *(P < 2.2e-16)*

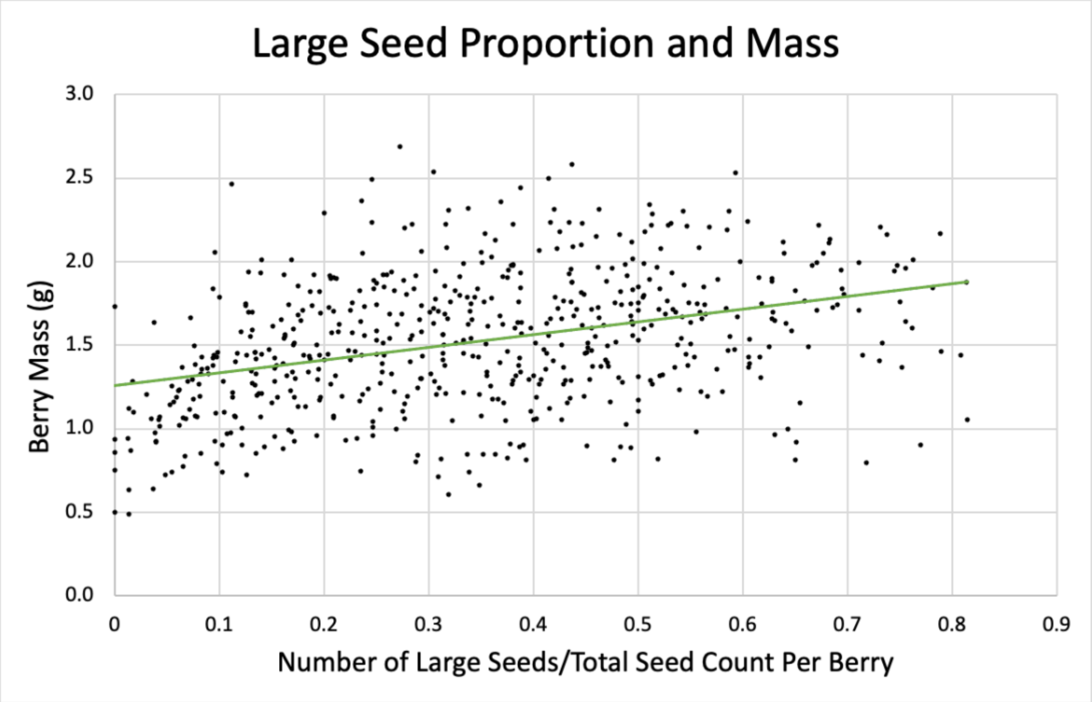

Figure 14: Correlation of proportion of large seeds per berry to its mass. Green line = line of best fit. $R^2 = 0.1256$ *(P < 2.2e-16)*

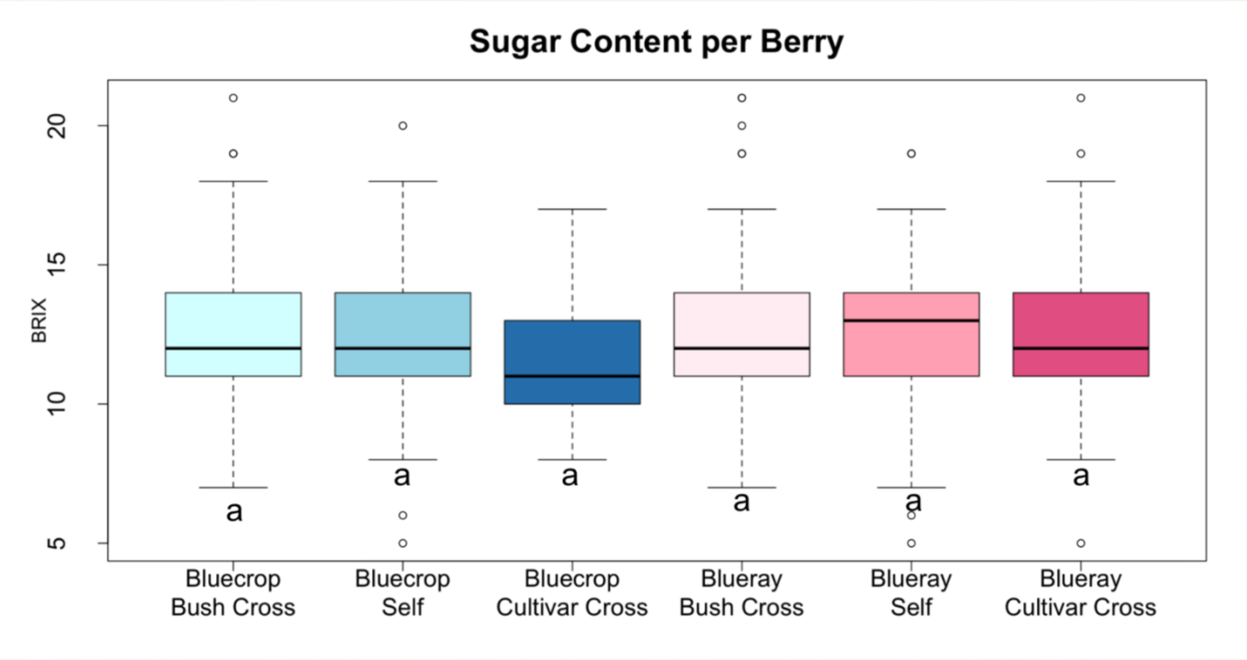

Figure 15: Sugar content (measured by the amount of soluble solids in berry juice) per berry in each treatment group. Blue = Bluecrop, Pink = Blueray, Light = pollination from a different bush of the same cultivar, Medium = pollination from the same bush, Dark = pollination from a different cultivar (P = 0.32196)

Variable	**Model**
BRIX index	Basic linear regression model
Fruit Set	Bimodal (fruited or unfruited) model for summary data after being transformed logarithmically
Viable Seeds per Berry	Nonbinomial generalized linear model with a Poisson family function
Proportion of Seeds Viable	Basic linear regression model after using an arcsine transformation
Large Seeds per Berry	Basic linear regression model
Proportion Large Seeds	Basic linear regression model after using an arcsine transformation
Mass	Basic linear regression model

DISCUSSION

Outcrossing and selfing both have biological benefits to plants that can affect their fitness in the short and long term (Miller & Owens 2009). This study provides a greater understanding of the mixed mating system found in blueberries. Outcrossing did not improve most Blueray and Bluecrop blueberry traits. In fact, within-cultivar crossing resulted in smaller berries than cultivar crossing or selfing in both Blueray and Bluecrop. Outcrossing did not significantly differ from selfing in viable seed and sugar production, or total berries produced. However, between-cultivar crossing did improve fertilization and resulted in more large, viable seeds. Large seeds are further along in the growth process and provide a better chance of reproduction (Bell 1957). Additionally, there was a positive relationship between large seeds and berry mass, which could explain the differences in mass between within-cultivar crosses and between-cultivar crosses. Within-cultivar crossing resulting in the statistically smaller berries could be explained by outcrossing resulting in smaller blueberries than selfing, but between-cultivar crossing also results in larger seeds.

Berry production and fruit quality have been enhanced by outcrossing in some studies (Mackenzie 1997, Bieniasz 2007) but not in others (Dogterom et al. 2000, Ehlenfeldt 2001). The variation in response to outcrossing may be cultivar specific. Cross pollination from some cultivars does not affect Bluecrop blueberry traits (Dogterom et al. 2000), while cross pollination from other cultivars does (Ehlenfeldt 2001). Across multiple cultivars, differences in large seeds (defined as plump and sound) account for a small percentage of berry mass variation (Eaton 1967). It is likely that blueberry plants invest more in fruits that contain a greater number of fertilized seeds, resulting in larger berries (Lee 1988).

Cultivar genotypes being compatible with some, but not all others, is not exclusive to Blueberries. For example, responses in Italia grapes depended on the variety of grapes with which they were crossed. One grape variety, 1103 P, increased the viable seed count and weight of Italia grapes, but no effect was found when Italia was crossed with 140 Ru (Sabir 2011). In apples, some varieties produce larger fruit with pollen from another cultivar, but other varieties fail to produce fruit at all when outcrossed (Wicks 1918). For oranges, crossing some cultivars with Valencia Late oranges produced a higher fruit set. Those same cultivars, however, had the greatest fruit mass when crossed with Rhode Red Valencia oranges (Yıldız & Kaplankıran 2017). In many different plants, outcrossing effects can be dependent on the specific cultivars being crossed. Cellular compatibility or incompatibility controls whether pollen can fertilize ovules or not, and genetics may play a role (Hiscock & Allen 2008).

Blueray and Bluecrop originated from the same stock and exhibit similar flowering times, berry masses, and sweetness, so they could be genetically very similar. Bluecrop, the older variety in this study, was developed in 1934 by crossing the same four cultivars that ultimately created Bluerays. Blueray was first developed in 1941 when the cultivar GM37 was crossed with a CU5. Those two cultivars originally were from Jersey, Pioneer, Stanley, and June cultivars (Campa & Ferreira 2018). If Blueray and Bluecrop are very genetically similar, selfing and outcrossing would not be that different. Yet, despite their common ancestry, they are different cultivars, so their genetics are not identical. There is still genetic variation, but that variation may not account for the results I observed.

The benefits and costs of selfing and outcrossing are of long-standing debate (Miller & Owens 2009). As stated previously, selfing can lead to inbreeding depression, so selfing may be evolutionarily selected against. Outcrossing, however, is not always possible. If there is a lack of flying pollinators, outcrossing is not advantageous, even with its negative effects. Plants have evolved many ways of enforcing self or cross pollination to ensure fertilization. Self-incompatibility, a trait in some angiosperms, can be determined by S-RNase. This enzyme produces highly diverse proteins that all function the same but have different genetic markers depending on the individual plant they are from (Lee et al. 1994). If similar absolute outcrossers are being crossed, the pollen and piston will have similar proteins and consequently, the pollen will be selected against to prevent inbreeding (Martin et al. 2004). The plant will be fertilized with less similar pollen if it is available. The delayed development of male versus female sex parts within flowers promotes outcrossing (Pang & Saunders 2014). Flowers may develop female organs first, then male organs as they mature (protogyny) so they can only be pollinated early in their life cycle, then produce pollen later. In this case, pollen that lands on the stigma is more likely to come from another flower during the female phase (Pang & Saunders 2014).

Delayed selfing is a mechanism plants can use to ensure pollination (Goodwillie & Weber 2018). This method utilizes both outcrossing and selfing in succession, to reduce the impact of inbreeding depression while still maximizing fruit and seed production. Plants that depend on this system have adaptions to promote selfing such as corolla abscission, reduced herkogamy, style curvature, and more, which all occur after outcrossing is no longer rewarding (Goodwillie & Weber 2018). One study showed that out of 345 species (of 78 different families), 42% utilized pollination via both selfing and outcrossing (Barrett 2014). Another study of 741 populations from 105 species found that 63% used both pollination types (Whitehead et al. 2018). It is estimated that only 10-15% of angiosperms predominately use selfing (Write et al. 2013). In general, it is much more common for a plant to have a mixed mating system than a singular one (Vogler & Kalisz 2001).

The results from my research must be interpreted with an awareness of certain caution. My study compares selfing, within-cultivar, and between cultivar-crossing but did not include an open control due to time constraints. An open control would consist of unbagged branches on both Blueray and Bluecrop. The pollen would not have been manipulated by hand, but instead by natural insects. This would have added an estimate of how well the bushes were naturally producing without manipulation. Had I included an open pollination control, there would also be a check on how realistic the results were compared to naturally occurring pollination.

If an open control resulted in significantly different blueberries, it would demonstrate that the act of bagging and/or hand pollinating created differentiation in resulting berry traits. It could show that the amount of pollen being transferred by hand was not equivalent to the natural pollinators. In fact, one study on carpenter bees found that hand pollination caused significantly greater fruit set, berry weight, berry diameter, peel weight, number of seeds, and juice yield (Barrera Jr et al. 2021). Although, the lack of a control treatment may cause misleading results. A more reliable methodology would incorporate both direct (comparing one treatment to another) and indirect evidence (comparing a treatment to a control group) of significance (Caldwell et al. 2005). A control could also demonstrate if pollinators were limiting. If pollinators were limiting, hand pollination would have enhanced fruit and/or seed set over open pollination controls.

Furthermore, a constraint to this study was the usage of one bush for three treatments. Ideally, a study on pollination effects would incorporate an entire bush for each treatment, but as plant size increases, this becomes increasingly difficult, and subsections must be used (Wesselingh 2007). The design used here accounted for variation caused by the bushes themselves (if one had more weeds, an infection, etc.), but not how the treatments themselves could have impacted one another. If a treatment branch led to a higher production rate of seeds or larger berries, this could have pulled resources away from another treatment branch, inflating the difference in reproduction (Knight et al. 2006). Wesselingh (2007) argues that additional methodology must be included to counteract this problem by establishing the extent to which resource allocation plays a role. Suggested approaches if re-allocation is significant include manipulating resource availability by removing leaves or developing berries, and enclosure of pollinators in cages around inflorescence instead of hand pollinating to better mimic what the surrounding inflorescence experience (Wesselingh 2007). Caging pollinators on plants, although more complicated to manage crossing, would also remove the effects of hand pollinating, but could change how much pollen was delivered and from where. Additionally, I looked at only a small fraction of berries per bush, which was not proportional to the total number of berries. Berry mass of each bush and tradeoffs between size and number were not captured. However, this is the first study on Blueray's outcrossing vs selfing, so my findings can serve as a starting point for future research.

Although the difference in mass was only 0.1 grams, if a blueberry farmer wanted to optimize their berry mass, they should consider planting Blueray and Bluecrop interspersed. Since berry size increased when these two cultivars are out-crossed or selfed, but not when they were crossed within cultivars, it is important to set up a system where the only crossing that is happening is between-cultivar crossing. A possible way to do this would be to alternate Blueray and Bluecrop so that no two cultivars next to each other are the same. This would reduce the likelihood that optimally foraging bumblebees, the main blueberry pollinators, would transfer pollen from one bush to another of the same cultivar (Thomson & Plowright 1980, Sabine et al. 2020). A larger mass of berries, even with small differences, would mean greater food production per bush, with minimal loss to sweetness (as BRIX was unaffected). This could bring in more money for the same amount of work, simply by adjusting the farm layout.

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APPENDICES

Key

Cross = pollination type

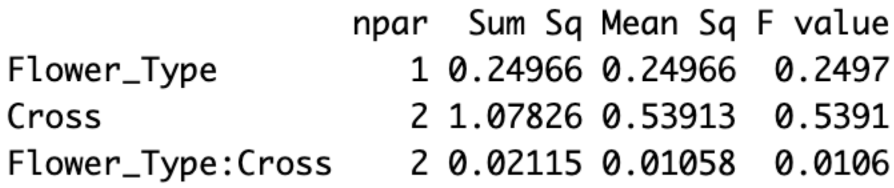
Flower_Type = cultivar variety

Self = self pollinated

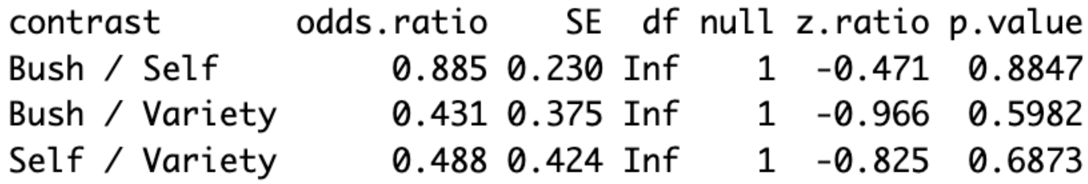
Bush = bush crossed

Variety = cultivar crossed

FRUIT SET

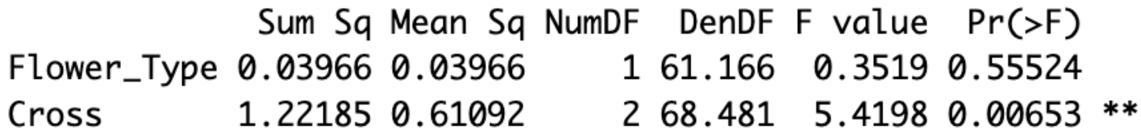


```
                  npar  Sum Sq Mean Sq F value
Flower_Type          1 0.24966 0.24966  0.2497
Cross                2 1.07826 0.53913  0.5391
Flower_Type:Cross    2 0.02115 0.01058  0.0106
```

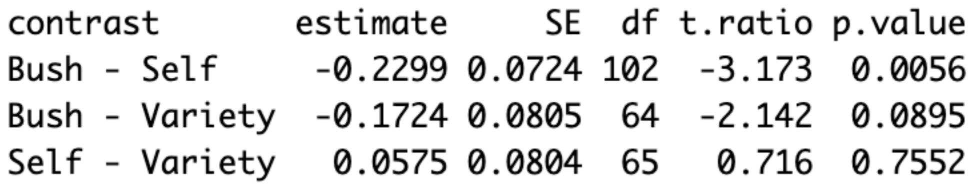


```
 contrast       odds.ratio    SE  df null z.ratio p.value
 Bush / Self         0.885 0.230 Inf    1  -0.471  0.8847
 Bush / Variety      0.431 0.375 Inf    1  -0.966  0.5982
 Self / Variety      0.488 0.424 Inf    1  -0.825  0.6873
```

BERRY MASS

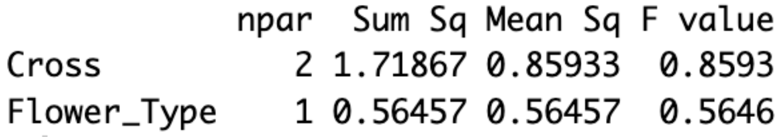


```
             Sum Sq Mean Sq NumDF  DenDF F value  Pr(>F)   
Flower_Type 0.03966 0.03966     1 61.166  0.3519 0.55524   
Cross       1.22185 0.61092     2 68.481  5.4198 0.00653 **
```

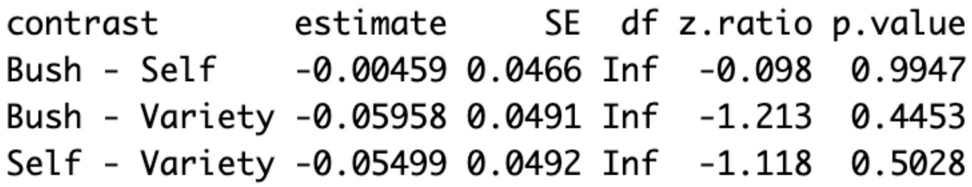


```
 contrast       estimate     SE  df t.ratio p.value
 Bush - Self     -0.2299 0.0724 102  -3.173  0.0056
 Bush - Variety  -0.1724 0.0805  64  -2.142  0.0895
 Self - Variety   0.0575 0.0804  65   0.716  0.7552
```

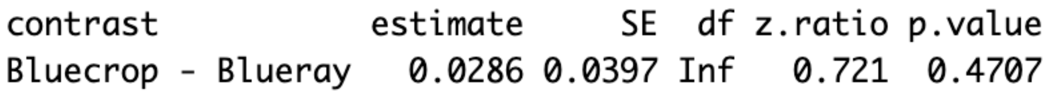
VIABLE SEED COUNT



```
            npar  Sum Sq Mean Sq F value
Cross          2 1.71867 0.85933  0.8593
Flower_Type    1 0.56457 0.56457  0.5646
```

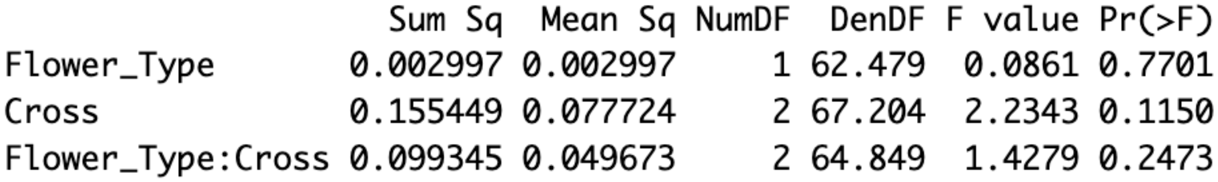


```
contrast        estimate     SE  df z.ratio p.value
Bush - Self     -0.00459 0.0466 Inf  -0.098  0.9947
Bush - Variety  -0.05958 0.0491 Inf  -1.213  0.4453
Self - Variety  -0.05499 0.0492 Inf  -1.118  0.5028
```

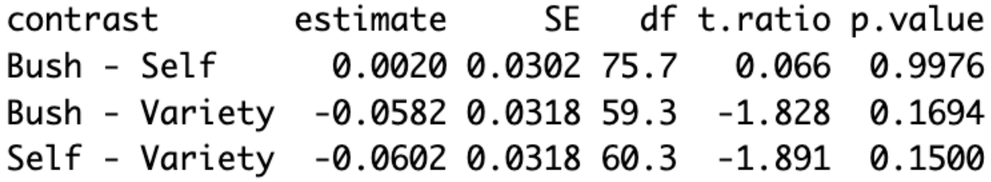


```
contrast            estimate     SE  df z.ratio p.value
Bluecrop - Blueray    0.0286 0.0397 Inf   0.721  0.4707
```

VIABLE SEED PROPORTION

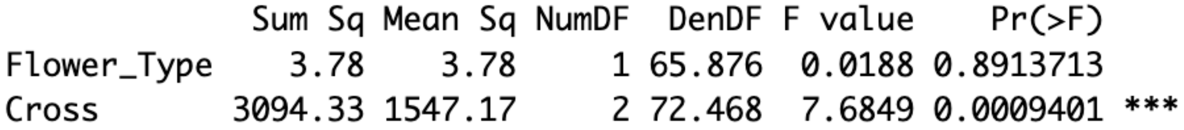


```
                     Sum Sq  Mean Sq NumDF  DenDF F value Pr(>F)
Flower_Type        0.002997 0.002997     1 62.479  0.0861 0.7701
Cross              0.155449 0.077724     2 67.204  2.2343 0.1150
Flower_Type:Cross  0.099345 0.049673     2 64.849  1.4279 0.2473
```

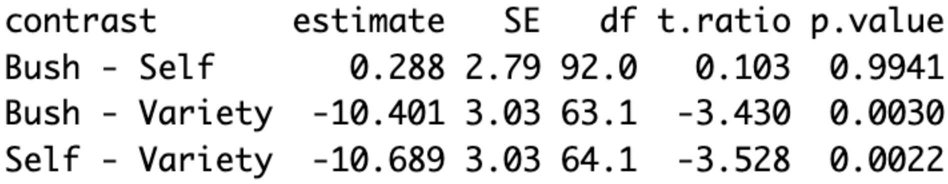


```
contrast        estimate     SE   df t.ratio p.value
Bush - Self       0.0020 0.0302 75.7   0.066  0.9976
Bush - Variety   -0.0582 0.0318 59.3  -1.828  0.1694
Self - Variety   -0.0602 0.0318 60.3  -1.891  0.1500
```

LARGE SEED COUNT

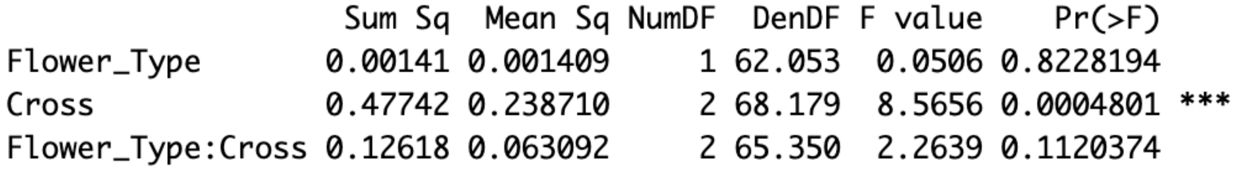


```
             Sum Sq Mean Sq NumDF  DenDF F value    Pr(>F)
Flower_Type    3.78    3.78     1 65.876  0.0188 0.8913713
Cross       3094.33 1547.17     2 72.468  7.6849 0.0009401 ***
```

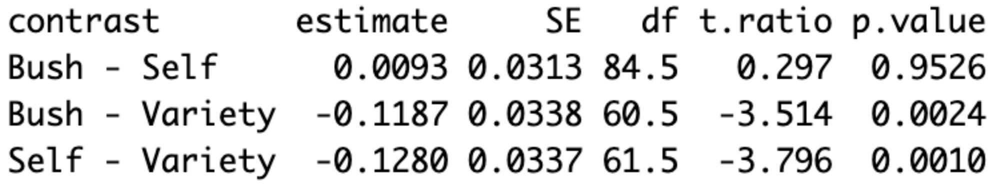


```
contrast        estimate   SE   df t.ratio p.value
Bush - Self        0.288 2.79 92.0   0.103  0.9941
Bush - Variety   -10.401 3.03 63.1  -3.430  0.0030
Self - Variety   -10.689 3.03 64.1  -3.528  0.0022
```

LARGE SEED PROPORTION

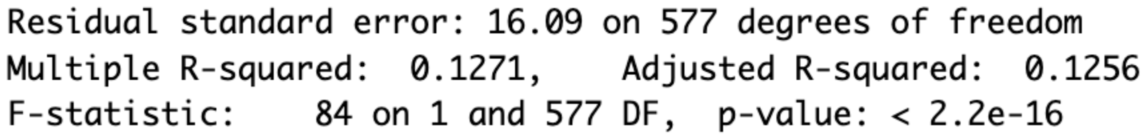


```
                    Sum Sq  Mean Sq NumDF  DenDF F value    Pr(>F)    
Flower_Type        0.00141 0.001409     1 62.053  0.0506 0.8228194    
Cross              0.47742 0.238710     2 68.179  8.5656 0.0004801 ***
Flower_Type:Cross  0.12618 0.063092     2 65.350  2.2639 0.1120374    
```

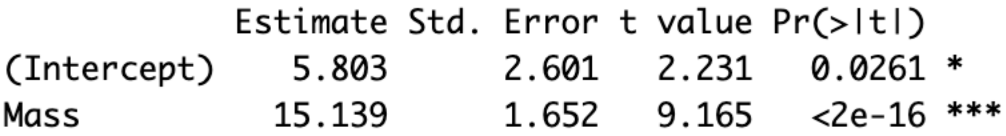


```
contrast        estimate     SE   df t.ratio p.value
Bush - Self       0.0093 0.0313 84.5   0.297  0.9526
Bush - Variety   -0.1187 0.0338 60.5  -3.514  0.0024
Self - Variety   -0.1280 0.0337 61.5  -3.796  0.0010
```

RELATIONSHIP OF MASS AND LARGE SEED COUNT

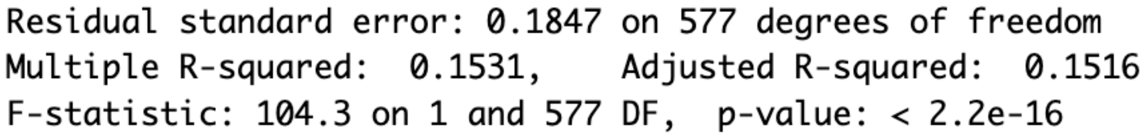


```
Residual standard error: 16.09 on 577 degrees of freedom
Multiple R-squared:  0.1271,    Adjusted R-squared:  0.1256 
F-statistic:    84 on 1 and 577 DF,  p-value: < 2.2e-16
```

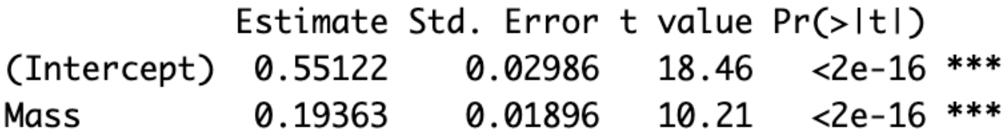


```
            Estimate Std. Error t value Pr(>|t|)    
(Intercept)    5.803      2.601   2.231   0.0261 *  
Mass          15.139      1.652   9.165   <2e-16 ***
```

RELATIONSHIP OF MASS AND LARGE SEED PROPORTION

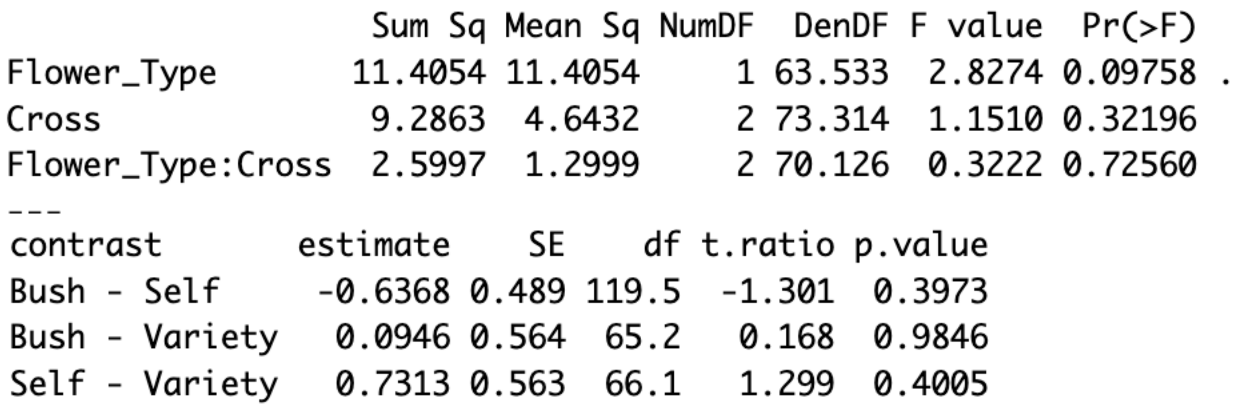


```
Residual standard error: 0.1847 on 577 degrees of freedom
Multiple R-squared:  0.1531,    Adjusted R-squared:  0.1516 
F-statistic: 104.3 on 1 and 577 DF,  p-value: < 2.2e-16
```



```
            Estimate Std. Error t value Pr(>|t|)    
(Intercept)  0.55122    0.02986   18.46   <2e-16 ***
Mass         0.19363    0.01896   10.21   <2e-16 ***
```

SUGAR CONTENT



```
                   Sum Sq Mean Sq NumDF  DenDF F value  Pr(>F)
Flower_Type       11.4054 11.4054     1 63.533  2.8274 0.09758 .
Cross              9.2863  4.6432     2 73.314  1.1510 0.32196
Flower_Type:Cross  2.5997  1.2999     2 70.126  0.3222 0.72560
---
contrast       estimate    SE    df t.ratio p.value
Bush - Self     -0.6368 0.489 119.5  -1.301  0.3973
Bush - Variety   0.0946 0.564  65.2   0.168  0.9846
Self - Variety   0.7313 0.563  66.1   1.299  0.4005
```