Dihydroflavonol 4-reductase gene expression and color change in the protandrous flowers of *Saponaria officinalis*

By

Taylor Renee York

Honors Project Thesis Submitted in Partial Fulfillment

Of the Requirements for a Baccalaureate Degree "With Distinction"

In the Ron and Laura Strain Honors College of

THE UNIVERSITY OF INDIANAPOLIS

May 2020

Faculty Advisor: Sandra L. Davis, Ph.D.

Executive Director of Honors: James B. Williams, Ph.D.

Abstract

Flowers of *Saponaria officinalis* exhibit protandry and floral color change: in early stages of anthesis, flowers bloom white and are male, then the petals turn pink as they transition to female. The depth of pinkness is determined by genetic and environmental factors. The pink color is due to the accumulation of anthocyanidin pigments, which are created through a biochemical pathway, each step catalyzed by enzymes, whose production is controlled by genes. Variation among plants in expression of these genes may lead to differences in final color or the rate that color change occurs. If these morphological differences result in fitness variation among individuals, the differences in gene expression may be subject to natural selection. Therefore, the pattern and extent of gene expression may represent an adaptive trait. This experiment quantified *Dihydroflavonol 4-reductase gene (DFR)* expression using quantitative polymerase chain reaction. First, gene expression was measured in samples obtained from previously collected late-stage female flowers, all which had their color quantified. Next, *DFR* expression was measured in flowers collected at five different life stages from ten different plants. We found *DFR* expression has no significant correlation with the level of pinkness reached in the female stage flower but does significantly increase as plants change from male to female.

Table of Contents

Acknowledgments

I would like to thank my parents for pushing me into accepting the invitation to join the Honors College at the University of Indianapolis and encouraging me to continue each year. I would like to thank Dr. Davis for working with me continually for the past two and half years. Finally, I would like to thank the Honors College for supporting me through my undergraduate work and funding my research.

List of Tables

List of Figures

Statement of Purpose

The purpose of this study was to contribute to the understanding of where in the anthocyanidin production pathway regulation of pink flower pigment production occurs. It has already been shown that the gene for anthocyanidin synthase (*ANS*), which acts later in the biochemical pathway, does not have a high level of expression until the flowers begin to change color, so I wanted to see if *DFR,* which acts earlier in the biochemical pathway, shows the same expression pattern.

The significance of this experiment is that it is part of a larger research program that seeks to answer the question: is the color change seen in the flowers of *S. officinalis* an adaptive trait? Adaptation is the result of natural selection. If natural selection is at work, individuals that have gene variants that confer a phenotype that increase their fitness, pass those genes to the next generation at a higher rate than those that lack those variants. Over time, this theoretically weeds out most of the "bad" genes and leaves the "good" genes in the population. Color change may be an adaptive response to many things, one of which is pollinators. By changing colors, these flowers could be signaling to pollinators which flowers contain pollen (the white male flowers), and which flowers do not (the female pink flowers). This may increase visits to the male-phase flowers which would benefit due to increased pollen dispersal over visits to female-phase flowers which would not benefit as greatly since s single pollinator visit could provide a large enough pollen deposit to fertilize all the ovules (Willson, 1994). Color change may also be an adaptive response to environmental conditions. Flowers of *S. officinalis* turn pinker when exposed to the sun (Jabbari *et al*., 2012), and anthocyanins may serve as protection against damage due to UV light as they have antioxidant properties (Glover & Martin, 2012). When in the biosynthetic pathway anthocyanin production is regulated will help to determine which genes

may be involved in governing differences between individuals in their color and how quickly plants can respond to environmental stimuli.

Using quantitative polymerase chain reaction (qPCR), I measured the expression of the gene that codes for the enzyme, Dihydroflavonol 4-reductase (DFR), an enzyme that is active in the anthocyanidin metabolic pathway. This pathway is a series of biochemical reactions that produce anthocyanins, the pigments that produce the pink color in flowers of *Saponaria officinalis*. To examine whether differences in how pink the flowers of individual plants become are associated with differences in *DFR* expression, I measured *DFR* expression by qPCR, then compared *DFR* expression to pinkness index in these flowers. In a second experiment, to see if the increase in color seen during the development of flowers was associated with an increase in *DFR* expression as they age, I again used qPCR to compare *DFR* expression across maturation stages in the *Saponaria officinalis* flowers.

Introduction: *Background Information*

The angiosperm *Saponaria officinalis* shows two distinct traits that are of interest to plant reproductive biologists: protandry and floral color change. Protandry is a reproductive trait whereby the flower's male reproductive organs, the stamens, mature and senesce before the female reproductive structures, the pistils, become receptive. This reduces the amount of self-fertilization that can occur, which may be beneficial because self-fertilization decreases the genetic diversity of the offspring produced, which carries negative evolutionary consequences. Therefore, protandry is considered an adaptation that increases outcrossing (Lloyd & Webb, 1986). However, it is pointed out by Lloyd and Webb (1986) that protandry may not have evolved solely for this reason.

The second feature of interest in *Saponaria officinalis* is the floral color change. Individual flowers first bloom as white, and over a few days, the petals turn pink. This color change is due to an accumulation of anthocyanin pigments in the petals (Jabbari *et al*., 2012). There are some environmental factors that affect how pink the flower turns. For example, plants mostly in the sun have flowers that turn pinker than those growing in the shade (Jabbari *et al*., 2012)**.** Furthermore, Davis *et al*. (2014) found heritability contributes to color change, as clonal lines differed significantly in the final pinkness level that flowers reach. Combined with the protandrous nature of the flowers, this means that male-phase flowers are still clearly white in all plants, but they transition to pink as they switch to female-phase. Therefore, the degree of pinkness a female-phase flower reaches depends on the genetic make-up of the parent plant which produce it and the environment in which that plant is growing.

The evolutionary significance and molecular mechanisms underlying floral color change in *Saponaria officinalis* are still largely unknown. One possibility is that the increased anthocyanin levels are due simply to the side effects of aging (Davis & Jones, 2008; Jabbari *et al*., 2012), as many flowers follow this pattern of color change during senescence (Delph and Lively, 1989). Another possibility is that *Saponaria* growing in the shade do not turn as pink as those in the light since the *Saponaria officinalis* growing in the sun may be light stressed and produce anthocyanins in response (Nawrocki, 2013). Anthocyanins are also used as antioxidants to protect tissues from oxidative stress that is caused by light under stressful conditions (Glover & Martin, 2012). Yet another possibility is that floral color change has been naturally selected to attract pollinators to individual plants but provide the insects with a signal to avoid older, non-reproductive flowers within the plant. Makino and Ohashi (2016) did not work with the *Saponaria officinalis* but did work with other types of color-changing flowers. They

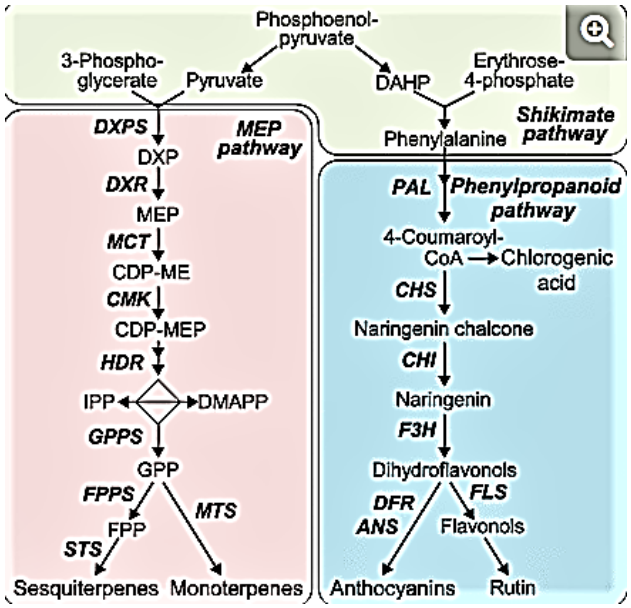
demonstrated that full-color changes give honest signals to pollinators with spatial memory to help with plant-level avoidance, as plants that give pollinators rewards are visited more than plants that do not give rewards. They also suggest if the plant changes color, the pollinators do not remember that the plant does not give a reward.

A unique feature of *Saponaria officinalis* is that because the changes in color occur during its transition from male and female phases, flowers are still sexually active after the color change. This suggests that the color change is not simply due to a side effect of senescence. Researchers demonstrated that there is a difference between male- and female-phase flowers when it comes to how many pollinators visit the flowers. Pollinators clearly favored white male-phase flowers over pink female-phase flowers (Nawrocki, 2013; Davis *et al.*, 2014). In this sense, the color change may be favored by selection as a mechanism to increase the visitation of male-phase flowers over female-phase flowers because female-phase flowers do not need as many insect visits. They receive enough pollen grains to fertilize all their eggs, whereas male-phase flowers will increase pollen dispersal with increased pollinator visits (Willson, 1994).

Problem Statement

The color change in the petals of *S. officianalis* is caused by the production of pigments called anthocyanins, which have a complex, multistep pathway through which they are produced (Fig 1). Each step in the biochemical pathway that produces anthocyanins is catalyzed by a different enzyme. In turn, each of these enzymes is coded for by its own gene. For example, the enzyme anthocyanidin synthase (ANS) is coded for by the anthocyanidin synthase gene (*ANS*). Previous work by students under Dr. Davis's supervision have looked to see if the production of this enzyme correlated to the color of the *Saponaria officinalis* (unpublished data).

Figure 1: The Anthocyanin Pathway. From: Kang et al., 2014



In one study, 53 *Saponaria officinalis* female-phase flowers of different plants were collected by Dr. Davis in the summer of 2015, and it was recorded where the flower ranked on a pinkness index (PI). The pinkness index was measured by using a reflectometer to record the amount of light reflected from an individual petal across the visual light spectrum. This reflectance spectrum was then used to calculate a single number to quantify how "pink" a flower is (Fig 2). Once the phenotype had been recorded, the RNA was extracted from the petals of each flower and reverse transcription was used to create cDNA (complementary DNA) samples. Real-Time or Quantitative Polymerase Chain Reaction (qPCR) was then performed on each of the samples using primers specific for the *ANS* gene. This measures the concentration of messenger

$$PI = \frac{\overline{\%R_{540-575nm}}}{\overline{\%R_{455-480and650-700nm}}}$$

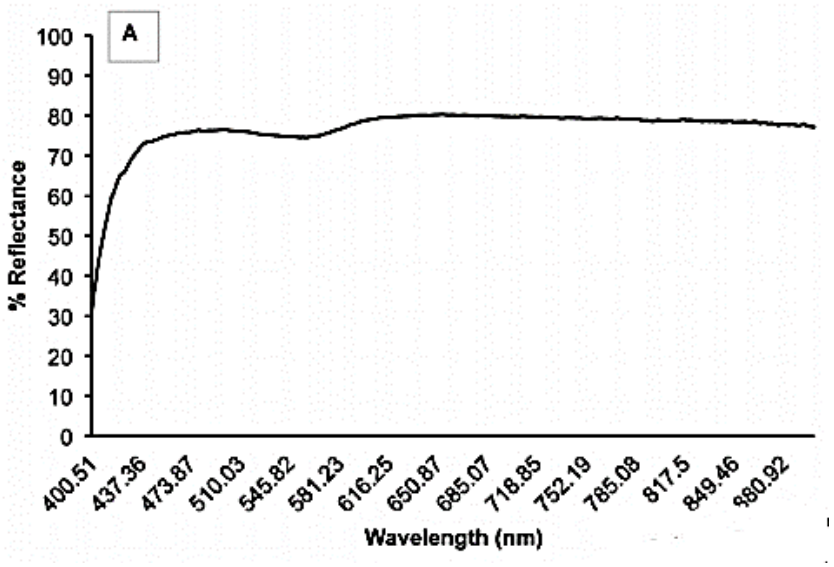


Figure 2: The equation to calculate the pinkness index and an absorbance spectrum (Davis et. al, 2014)

RNA (mRNA) for the *ANS* gene that was present in the original sample. Since the production mRNA is the first step in the expression of a gene, the amount of mRNA is used to quantify the level of expression of the gene. In this case, there was a significant correlation between PI and *ANS* expression, so that the higher the expression level of *ANS* in the flower, the higher they pink index for the flower (Eckert, unpublished data; Fig 3). This indicates that the differential expression of the *ANS* gene contributes to the final color of female-phase flowers.

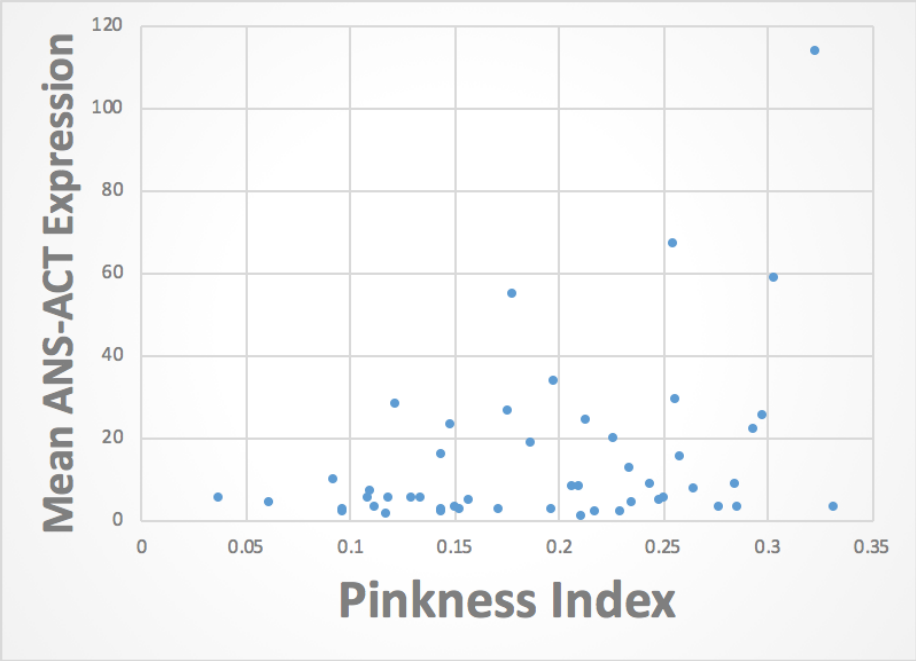


Figure 3: The correlation between *ANS* expression and pinkness. Eckert, unpublished Data.

In a second study, another student researcher determined if there was a significant difference between the sexual phases of the *Saponaria officinalis* flowers and the expression of *ANS.* She found that there was a significant difference in the expression of *ANS* in male-phase compared to female-phase flowers (Foy, unpublished data). Therefore, increases in ANS expression are also involved in the transition from white to pink as the flower ages.

For my project, I explored the connection between flower color and expression of a second gene, Dihydroflavonol 4-reductase (*DFR*), which codes for an earlier enzyme in the anthocyanin pathway. Theoretically, *DFR* and *ANS* should both show a similar relationship to color since they are found in the same pathway. However, Farzad *et al.* (2003) showed another story as they explain the color-changing in the *Viola*. They examined three genes in the *Viola cornuta*, chalcone synthase (*CHS*), *DFR,* and *ANS,* and examined how their expression changed over the lifetime of the *Viola cornuta* and in plants growing in different light conditions. They found *ANS* showed a dramatic increase in expression as the flower aged, but *CHS* and *DFR* were

differentially expressed in plants growing under differing light conditions. Therefore, the color change may be regulated by different gene combinations in the same biochemical pathway due to different stimuli.

For my Honors projects, I compared expression of the *DFR* gene to floral color change in the *Saponaria officinalis.* To carry out my experiment, I used the same 53 samples used in the previously described study, with the phenotypes known, and used qPCR to determine if, like *ANS, DFR* expression is correlated with how pink female-phase flowers become. I hypothesized that the higher the flower of the *Saponaria officinalis* was on the pink index, the higher the *DFR* gene expression should be. In the second part of my project, I used a different set of samples collected in 2016, also with known phenotypes, and again used qPCR to determine whether *DFR* expression differed between male- and female-phase flowers. I hypothesized that the later the phase the *Saponaria officinalis* was in, the higher the expression of *DFR* should be.

My findings showed that *DFR* does not have a statistically significant correlation with pinkness in the late female-phase of the *Saponaria officinalis*. This suggests that for the *S. officinalis*, *ANS* expression contributes more to the flower color than *DFR*. However, while DFR does not correlate with pinkness, it does significantly increase over the lifespan of the *Saponaria officinalis.*

Methods:

Correlation of DFR expression vs color of female-phase flowers

In 2015, Dr. Davis and colleagues collected 53 samples of late female-phase flowers of *Saponaria officinalis* from plants in three separate locations at the DePauw University Nature Park: an experimental sun-exposed garden plot, an experimental shade-exposed garden plot, and

natural populations found in the park (partially wooded). The reflectance spectrum of one petal was taken when the flowers were collected. The remaining petals were removed, and RNA extraction was done using the TRIzol™ protocol. Reverse transcription was conducted on each sample by a previous student to generate cDNA from each flower. The start of my experiment was to create one to ten dilutions of the 53 cDNA samples by taking five microliters of the cDNA and mixing it with 45 microliters of nuclease-free water. Of the 53 samples, five had been completely exhausted in other experiments, leaving me with a total of 48 samples. For each run of qPCR, I chose five of the 1:10 dilutions at random, until I ran all 48 samples. Then, I chose one of the undiluted samples that corresponded to one of the five dilutions to use to create a standard curve.

For each round of qPCR, I created two separate master mixes, one for *DFR* and one for *ACT*. Actin, or ACT, is a highly conserved, essential protein found in plant cells, produced on a continual cycle due to its many uses in the cell (Dominquez & Holmes, 2011). Due to its constant production, for this experiment, *ACT* is being used as a control, to correct for pipetting errors or loss of sample due to handling such small volumes. In each master mix, I added 16 microliters of SYBR Green™ and 16 microliters of the corresponding eight molar *ACT/DFR* primer mix. For the standard curve dilutions, I preformed six serial dilutions, starting with two microliters of the undiluted sample and 18 microliters of water. Reagents were then loaded into a 48-well plate to run in the real-time thermocycler. The plate layout shown in Table 1 was used for all qPCR runs, both in the first part of the experiment and the second part. Each well was filled with six microliters of either ACT or DFR primer mix and six microliters of a chosen sample as detailed below, for a total of 12 microliters in each well.

Table 1: Plate layout for all qPCR runs. Blue wells were those filled with ACT primer mix. Pink wells were those filled with DFR primer mix. The description in each well was what sample was placed in each well. Sample # are the randomly picked 1:10 dilutions for each session.

	1	2	3	4	5	6	7	8
A	Water	$1*10^{-1}$	$1*10^{-4}$	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
B	Water	$1*10^{-2}$	$1*10^{-5}$	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
C	Water	$1*10^{-3}$	$1*10^{-6}$	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
D	Water	$1*10^{-1}$	$1*10^{-4}$	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
E	Water	$1*10^{-2}$	$1*10^{-5}$	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
F	Water	$1*10^{-3}$	$1*10^{-6}$	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5

The plate was then placed in the thermocycler which was set to run the following protocol: 40 cycles of 1-minute denaturation at 95°C, 1-minute of annealing at 54°C, and 2 minutes of extension at 72 °C.

Changes in DFR expression as the flower transitions from male to female

For the second part of my project, I used samples collected by Davis and Dudle from July 19-22, 2016. They collected samples from 12 different *Saponaria officinalis* at the bud, early staminate, late staminate, early pistillate, mid pistillate, and late pistillate. Again, it was recorded where the flower ranked on the pink index. Once the phenotype had been recorded, the samples had their RNA extracted and quantified how much RNA was in the sample. Five mature buds were marked on the same day, so they would all open on the same day. One bud was taken, the reflection spectrum was recorded, and the petals were snap frozen in liquid nitrogen, then ground to a powder. The RNA was extracted using the TRIzol™ procedure. The flowers were tracked through their five stages, and at each stage, the reflectance was taken, as was a petal for RNA extraction. The TRIzol™ procedure was once again used to extract RNA. For this part of my

experiment, I started with these RNA samples instead of cDNA samples and therefore had to use reverse transcription to convert the samples to cDNA. Each reaction consisted of four microliters of RT Enzyme Master Mix (which was made with 52µl of RNase free water, 52µl RT buffer, 26µl of AMV reverse transcriptase), 2µl of oligo dT, 4µl of dNTP, and a volume of the RNA solution to contain 2µg of RNA and enough water to bring the total volume to 20µl. The samples were placed in a thermocycler program for 40 cycles of 1-minute denaturation at 95°C, 1-minute of annealing at 54°C, and 2 minutes of extension at 72 °C. Reverse transcription was conducted on 25 samples at a time, for 50 total samples. The resulting cDNA was then purified using a Qiagen PCR Purification Kit™. I then ran qPCR on each sample using the same procedure for part one.

To quantify gene expression, I took the average C_T (the cycle number that the sample started it's exponential phase) value for each sample using the *DFR* and *ACT* primers, and found the difference between them by subtracting the C_T value of *ACT* from the C_T value for *DFR*. Then, I calculated the "delta delta" value by taking the greatest difference found between the two (positive or negative), then taking the positive value of that difference and subtracting all differences by that value. Finally, I squared the final difference and that became the ratio that was used to measure *DFR* expression.

Results

Correlation of DFR expression vs color of female-phase flowers

It has already been shown that the environment does have an impact on how pink the late female-phase flowers get, particularly if they have been grown in shade or grown in the sun (Jabbari *et al*, 2012). However, the environment the flowers were growing in was taken into

consideration because we wanted a wide variety of pinkness values so that we could establish whether there is a correlation between *DFR* expression and how pink the late pistillate flowers become. The pinkness indexes from the flowers in this experiment ranged from 0.036472605 to 0.323499179.

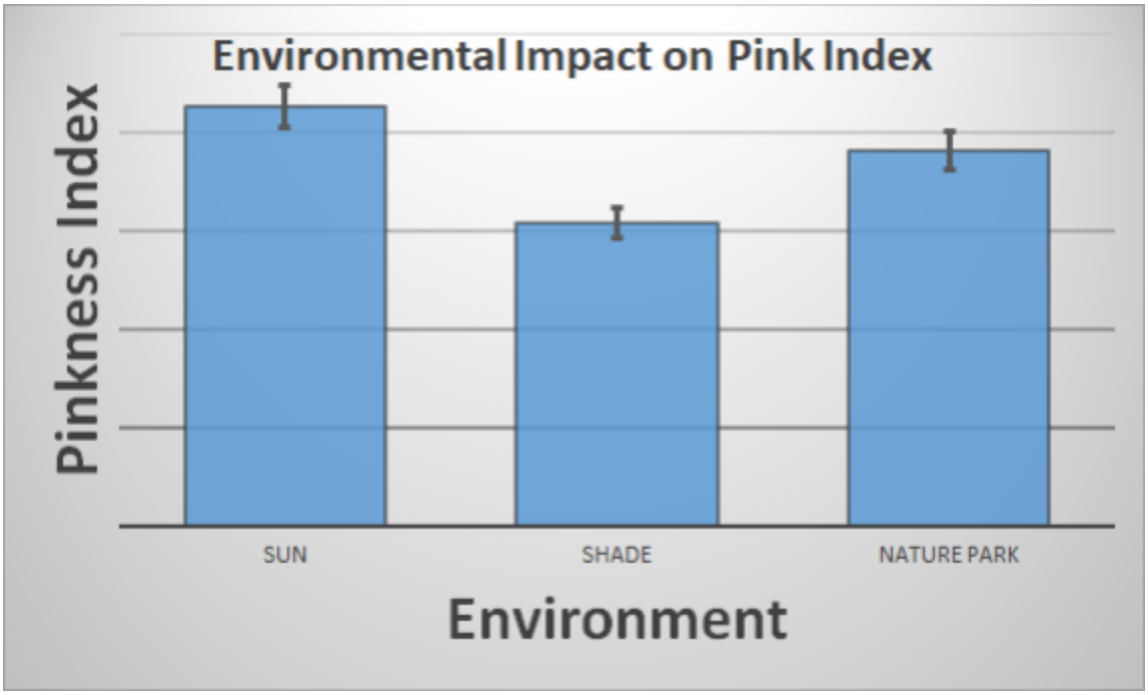


Figure 4: Pinkness Index of female-phase *S. officinalis* flowers collected from plants growing in different locations. This graph shows the relationship between how deep of a pink the late pistillate phase flowers get depending on their location from forty-eight different flowers.

There were 21 samples from the sun, 8 samples from the shade, and 24 samples from the nature park. While there might not be any significant difference between the groups, there does seem to be the right trend for the sun to have the highest amount of pink, the shade to have the least amount of pink, and the nature park to have an intermediate level (Fig. 4). Unlike previous studies, there was not a significant correlation between the environment the *Saponaria officinalis* was grown in and how pink the late phase pistillate flowers became ($P=0.072$, $R^2=0.034$;

Appendix II). I hypothesize that the lack of correlation is due to the small sample size from the shade samples.

There was no significant correlation between the expression of *DFR* and how pink the late pistillate-phase flowers became (P =0.373; Appendix III). There again does seem to be a small trend towards my hypothesis, shown in Figure 5, but there are many *DFR* expression values near 0 across all pinkness levels. This may indicate either our samples were of low quality, or our procedure was not sensitive enough to reliably make accurate measurements of *DFR* expression.

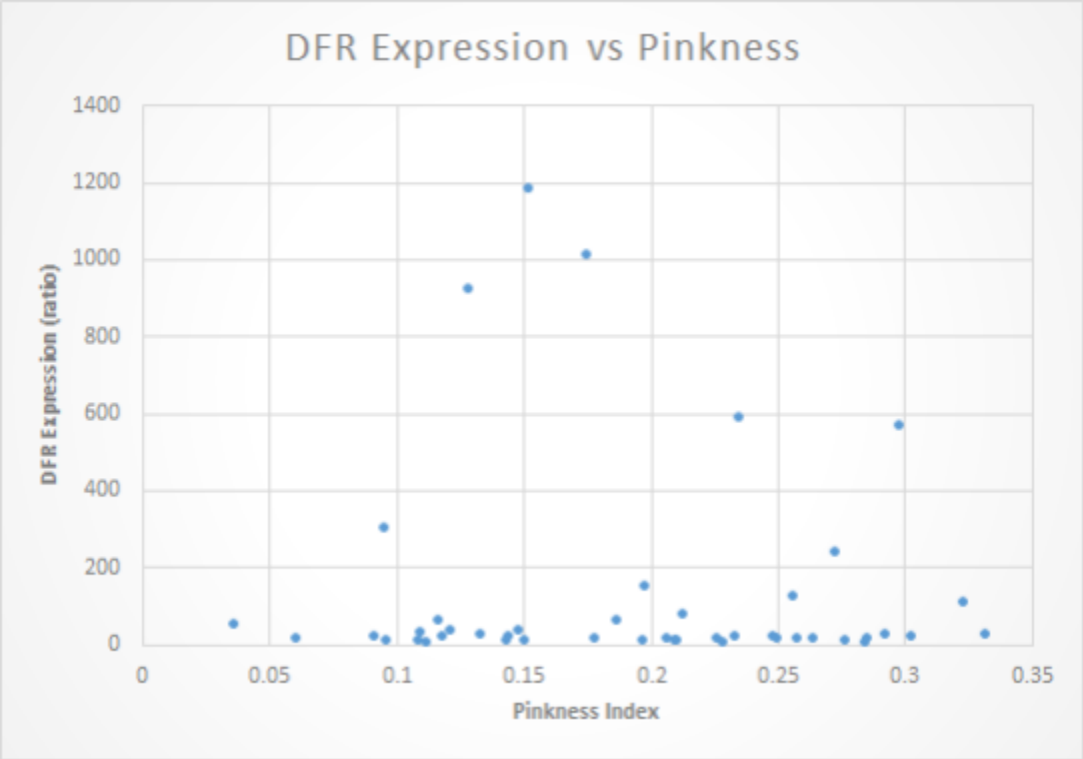


Figure 5: The lack of correlation between *DFR* expression and pinkness of late phase pistillate flowers. The relationship between *DFR* expression and the color of the pistillate phase flowers. As shown, the pinkness of the pistillate-phase flowers does not seem to be dependent on *DFR* expression in the *Saponaria officinalis*.

For both environmental factors and *DFR* expression, P values are above 0.05, meaning that neither impact how pink the late pistillate flowers of the *Saponaria officinalis* get. When looking at differences among the environments, we performed a one-way ANOVA, but when looking for the relationship, or lack of, between PI and DFR expression, we did a Pearson-Product Correlation. This statistical test tells us how much of a linear relationship the two variables have, which would be the relationship we are looking for if *DFR* expression impacted pinkness in the *Saponaria officinalis*.

Changes in DFR expression as the flower transitions from male to female

Davis and Dudle (2016) compared pinkness of S. officinalis to flower life stage. An ANOVA test determined there was a significant difference in how pink the *Saponaria officinalis* flower was and its life stage it was in. Since there was a significant difference, a Tukey Post hoc test was used to determine homogeneous subsets and establish which stages had significant differences in their pinkness index. The homogenous subsets broke down the stages into two groups. Group a consisted of Bud, Early Staminate, Late Staminate, and Early Pistillate. Group b consisted of Early Pistillate, Mid Pistillate, and Late Pistillate phases (Fig 6).

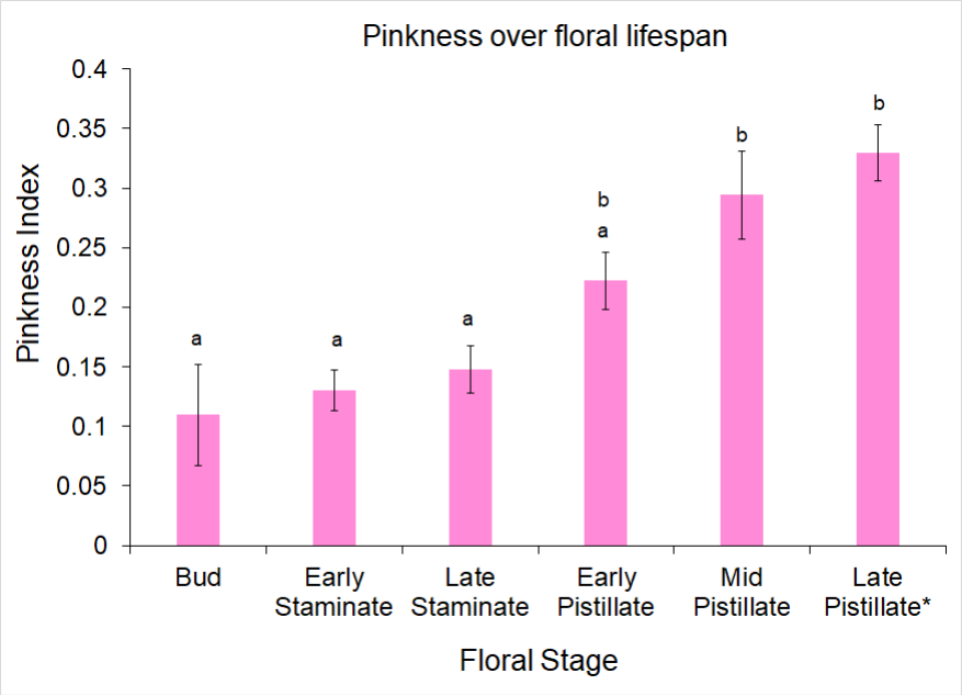


Figure 6: The average Pinkness Index of each floral stage for the *Saponaria officinalis*. This includes sixty-seven total samples: nine from bud stage, twelve from early staminate, twelve from late staminate, twelve from early pistillate, ten from mid pistillate, and twelve from late pistillate. The pinkness index of Group a was significantly different than that of Group b. (Davis & Dudle, 2016)

These two groups are broken down into the male phase of the *Saponaria officinalis*, and the female phase, with the very early female phase being the transition. While not statistically different, each stage progressing from early male to late female does seem to become pinker than the previous (Fig. 6). For my research, I quantified DFR expression using the same sample sets Davis and Dudle (2016) used in their research.

To determine if there was significant difference in *DFR* expression across the stages, again, an ANOVA with a post-hoc test was run. However, one outlier was found by the statistical program for the Early Staminate phase that we excluded, which gave us N = 8, 8, 8, 8,

8, 7 per stage. The ANOVA test showed there was a significant difference in *DFR* expression across the different stages (P=0.03; Appendix IV).

Next, the Tukey Post Hoc test was used to compare each stage to one another to show which stages were significantly different from one another. Stage A (bud) was significantly different from Stage B (early staminate) for *DFR* expression (P=0.010; Appendix V). Stage B, along with being different from Stage A, was also significantly different from Stage E (mid pistillate) (P=0.007; Appendix V).

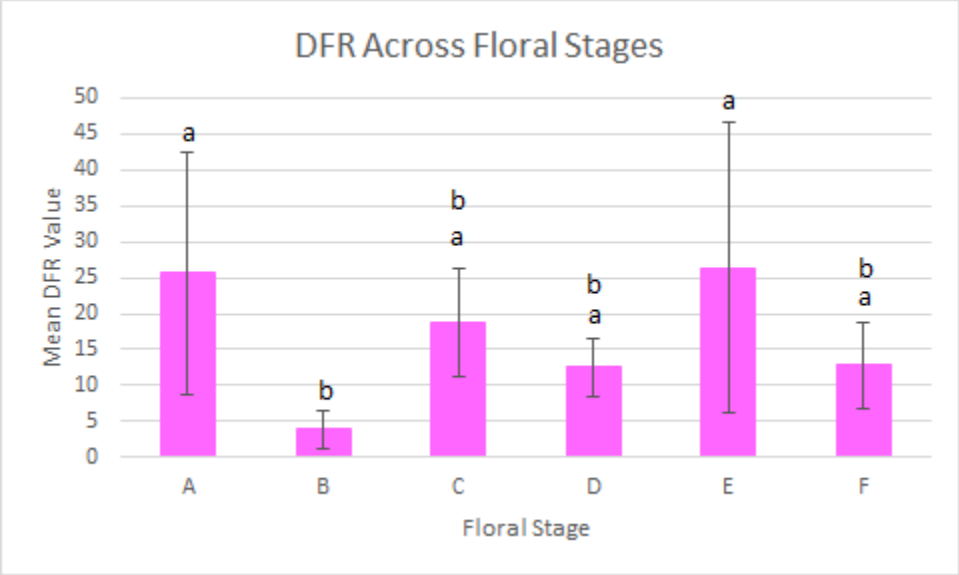


Figure 7: The average DFR expression found across the floral stages of the *Saponaria officinalis*. This analyzes forty-eight samples which includes eight bud (A), nine early staminate (B), eight late staminate (C), eight early pistillate (D), eight mid pistillate (E), and seven late pistillate (F). Group a has a significant difference in DFR expression than Group b.

Again, homogeneous subsets were determined and broke down the stages into two groups. Group a contained Early Staminate, Late Staminate, Early Pistillate, and Late Pistillate (Table 2, Fig 7). Group b contained Bud, Late Staminate, Early Pistillate, Mid Pistillate, and Late

Pistillate (Table 2, Fig 7). As Figure 7 shows, Early Staminate seems to have the least amount of *DFR* expression. Some stages are in both group a and b because they are not statistically different from any of the stages in group a, nor are they statistically different than any of the stages in group b.

Table 2: A Tukey HSD. This table was used to group the stages into subgroups. This is done by grouping together the stages that did not have significant differences from one another. With this data, there are only two subsets. Subset one has Early Staminate, Late Staminate, Early Pistillate, and Late Pistillate, all which do not have a significant difference between their expression of DFR. Subset two has Bud, Late Staminate, Early Pistillate, Mid Pistillate, and Late Pistillate, all which do not have a significant difference between their expression of DFR. A=bud, B=Early Staminate, C=Late Staminate, D=Early Pistillate, E=Mid Pistillate, F=Late Pistillate. Based on flower structure, A-C are male, and D-F are female.

			Subset	
	Stage	N	1	2
Tukey HSD[a,b,c]	B	9	5.0664	
	D	8	12.5125	12.5125
	F	7	12.7760	12.7760
	C	8	18.8026	18.8026
	A	8		25.6177
	E	8		26.4251
	Sig.		.202	.191

Means for groups in homogeneous subsets are displayed.
The error term is Mean Square (Error) = 137.263.

a. Uses Harmonic Mean Sample Size = 7.958.

b. The group sizes are unequal. The harmonic mean of the group sizes is used.

c. Alpha = 0.05.

An ANOVA test was used to determine if there was significant difference in *DFR* expression across the stages without including the bud phase. It was determined that without including the bud phase, there was still a significant difference across the stages (P= 0.002; Appendix VII). Since there was significant difference among the stages, a Tukey Post Hoc test was used to establish differences between individual stages.

These statistical tools (Appendix VIII) showed that Stage B (early staminate) was significantly different from both Stage C (late staminate) (P=0.05) and Stage E (mid pistillate) (P=0.001). The five different stages were broken up into two groups by a homogenous subset test. Group a consisted of Early Staminate, Late Staminate, Early Pistillate, and Late Pistillate (Fig. 8, Appendix VIII). Group b consisted of Late Staminate, Early Pistillate, Mid Pistillate, and Late Pistillate (Fig. 8, Appendix VIII). These groups are comparable to the groups determined earlier (Fig. 7), apart from the bud phase no longer in Group b.

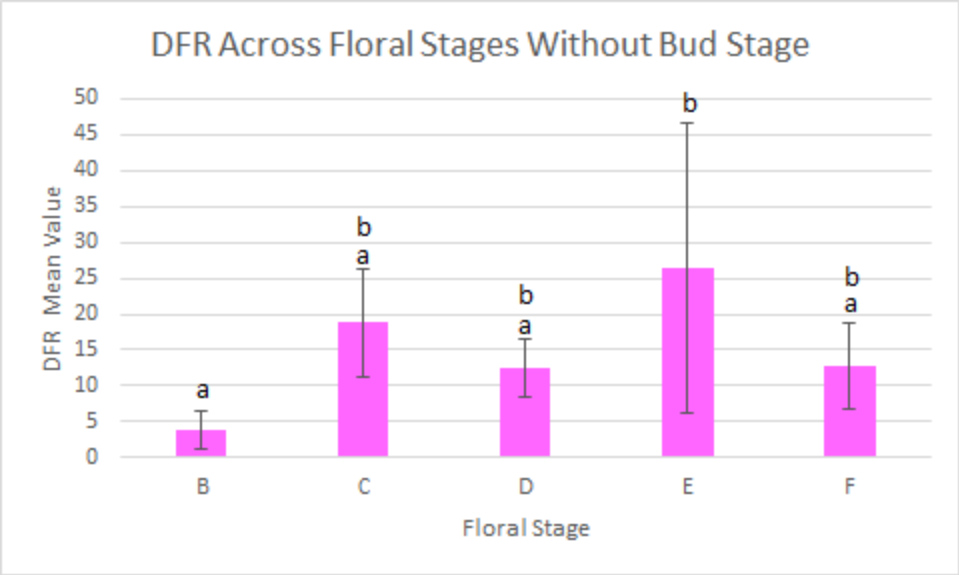


Figure 8: The average DFR expression found across the floral stages of the *Saponaria officinalis*. This analyzes forty samples which includes nine early staminate, eight late staminate, eight early pistillate, eight mid pistillate, and seven late pistillate. Group a has a significant difference in DFR expression than Group b. Data analyzed without Bud Phase.

I had hypothesized that the Bud stage would have the least amount of *DFR* expression, so I also analyzed the data without the bud phase, since the stage deviated from my original prediction. From my results, *DFR* expression does not influence how pink the late female stage flowers get. However, there is a significant change in *DFR* expression across the *Saponaria officinalis* lifetime.

Discussion

Correlation of DFR expression vs color of female-phase flowers

Although there was not a statistically significant difference in pinkness levels of the flowers collected from the various locations, previous studies have shown that sun exposure increases the color and concentration of anthocyanins in petals of *S. officinalis* (Jabbari *et al*,

2012). The purpose of collecting sun and shade flowers in this study was to provide a sample of flowers displaying a range of pinkness levels to look at gene expression in pistillate-phase flowers of *S. officinalis*. To that effect, in the 53 flowers collected, PI index ranged almost ten-fold, from 0.036472605 to 0.323499179. However, as we were interested in how *DFR* expression affects final color, we preferentially collected flowers across the pink color and collected only a few (8) samples that were from plants growing in the shade and had paler flowers. It is likely that the small sample size of shade flowers contributed to the lack of significant differences in PI across sun exposure levels, but the trend of shade plants having paler female-phase flowers than sun plants matched what we would predict based on previous studies.

Expression of the gene responsible for producing anthocyanidin synthase (ANS), an enzyme later in the anthocyanin pathway, has been shown to have a significant positive correlation with the pinkness level reached in a pistillate-phase flower (unpublished data). Theoretically, *DFR* should have shown the same correlation that *ANS* showed, since DFR is an earlier enzyme in the pathway, and therefore for there to be reactions that need ANS enzymes, there first needs to be products of reactions that DFR enzymes catalyze. However, *DFR* does not show significant correlation with pinkness (Fig 5). This does not support my hypothesis that the pinker the late pistillate-phase flowers were, the higher the *DFR* expression. Therefore, I revised my original hypothesis. I hypothesized that *ANS* expression is solely responsible for the differences among the *Saponaria officinalis* in their female-phase flower color.

This phenomena of *ANS* being the determining step for differences in color change among individuals is not just found in the *Saponaria officinalis*. Farzad *et al* (2003) showed that when comparing light and dark treatments, *ANS* had a large increase in expression in light treated flowers, while *DFR* production only had a slight increase in expression. This is like my

experiment as we chose flowers with a wide range of pinkness to see if *DFR* expression varied, including those grown in shade and those grown in sun, and we concluded there is no correlation between pinkness and *DFR* expression. Farzad *et al* (2003) similarly concluded that most of the floral color change from white to purple is dependent on changes in *ANS* expression, while *DFR* expression changes very little.

Changes in DFR expression as the flower transitions from male to female

In a previous study, Jabbari *et al.* (2012) found that as flowers of *S. officinalis* transition from white to pink, the petals show a significant increase in the concentration of anthocyanidin pigments, and this is also correlated with the gender change in the individual flowers. The purpose of this study was to further analyze the genetic mechanism that underlies this increase in pigment production. We confirmed that the flowers used in this study also showed this increase in pinkness (Fig. 6), as we found that the staminate-phase flowers had significantly paler flowers than the pistillate-phase flowers.

Since there was no correlation established between *DFR* expression and how pink flowers of the pistillate-phase of the *Saponaria officinalis* became, I did not expect to observe any difference between *DFR* expressions in the different stages of the *Saponaria officinalis*, even though I hypothesized earlier that there would be. While not every single stage had significant differences of *DFR* expression, there were a few stages that were different from one another. The bud stage did have a significantly higher level of *DFR* expression than the early staminate stage (Fig. 7). This is interesting, since we believed that anthocyanins were there mainly to change the color of the flower. However, it is not unprecedented, as Kawbata *et al*. (2009) found that there was a preferential accumulation of flavonoids (Fig 1 shows how flavonoids relate to

anthocyanins) at the bud stage and at anthesis in the *Eustoma grandiflorum*. However, this increase in *DFR* expression may not be related to color production. Flavonoids are a large group of organic molecules found in plants that are responsible for secondary metabolism, and one subgroup of these chemicals are anthocyanins (Davies *et al*., 2002). Flavonoids protect plants against various biotic and antibiotic stresses, including absorbing harmful UV radiation that can induce cellular damage (Samanta *et al.*, 2011). Samanta *et al.* (2011) concluded that some other functions include signaling molecules that activate pathways for root nodule growth and help promote germination and early plant growth. Similarly, flavonoids may be important to the bud stage to promote growth, provide signaling molecules for flower tissue formation, and possible light stress from the sun.

Since the higher expression of *DFR* in the bud stage was interesting, but may be unrelated to floral color, we decided to analyze the data without the bud stage. Not including the bud phase, the *DFR* expression of early staminate-phase showed a significant difference from the late staminate-phase and the mid pistillate-phase. The early staminate-phase and the mid pistillate-phase had consistent significant differences in *DFR* expression each set of data. Figure 8 clearly shows that the early staminate phase has the lowest amount of *DFR* production, whereas the mid pistillate phase has the highest amount of *DFR* production.

Farzad *et al.* (2003) found in Violas that transition from white to purple, *DFR* expression is not only present in all stages of the Viola's life but seems to be expressed at consistent levels across the floral lifespan. This is contrary to what I found, where *DFR* expression seems to peak as the color change begins. However, they also did find that *DFR* expression does increase over Viola's life by about 56% (Farzad *et al.*, 2003), a similar finding to the second part of my

experiment. This means that the pattern seen in *S. officinalis* in which *DFR* expression increases during floral ontogeny may be a widespread mechanism controlling floral color change.

Overall, I found that in the *Saponaria officinalis*, *DFR* expression does not seem to correlate with pinkness in late phase females, but *DFR* expression does seem to increase over the *Saponaria officinalis* lifetime. However, before researchers move forward, they should revisit this experiment and make a few improvements before making any final conclusions. For the first part of this experiment, larger and equal sample sizes should be collected from the *Saponaria officinalis* in sun, shade, and wild locations. This may give researchers a better idea on whether *DFR* expression has any impact on pinkness for female phase flowers. For the second part of this experiment, again, doing the experiment with a larger sample size may help solidify whether DFR expression shows a similar pattern to what was found in this study. QPCR also has many steps with micro amounts of liquids being transferred, meaning that there is plenty of room for error. There is also the possibility of lost or damaged DNA and RNA, so repeating this study all around would be a good idea.

If a general increase in *DFR* as flowers develop is observed again, more research should be done to reveal why only two phases of six are significantly different from one another. One observation, made by reviewers, is stages A, C, and E had higher DFR production than B, D, and F. Stage A could be considered a pre-B, Stage C could be considered a pre-D, and Stage E could be considered pre-F. This could correlate with the flower first blooming as a male (A to B), the flower losing its male reproductive organs and gaining it's female reproductive organs (C to D), and the maturation of the female flower (E to F). Further research could investigate whether these changes can be linked to *DFR* production, or perhaps they will find other reasons why these three stages seem to have higher production of *DFR* than the other three.

Reflection

The original goals of this project were to learn the technique of qPCR, to present my findings in an oral presentation, and to achieve significant results. I fully met my goal with learning how to do qPCR, and I also achieved significant results in both portions of my project, as both parts of my experiment contributed to the larger picture of understanding the color change of *S. officinalis*. However, I was not able to orally present my project due to the coronavirus pandemic preventing in person meetings. However, I will still be turning in a poster to the Biology department and a manuscript to the Honors College, so my findings are not completely unpresented, just in a different format than originally intended.

There were two main difficulties that I had to overcome during this project. First, very early on, the *DFR* primers did not seem to be working, even though they were indeed amplifying the correct gene. However, this was probably due to my lack of experience of running qPCRs, as just a drop of extra or less solution will mess up findings, and by the time I was ready to run actual samples, all the kinks seemed to have worked out. Secondly, the -80℃ freezer in the Biology department malfunctioned and the samples that I was using for the life stages portion of my research were ruined. Luckily, DePauw University had some RNA versions of the samples I was to use, so I was able to carry out the second portion of my research.

This project was personally important to me as I am now one step closer to graduation, something to which I have been looking forward. I did not find much interest in my high school graduation, but as my senior year has come and gone, I find myself looking ever so forward to my college graduation, something that is sure to be ten times more special, especially since I will now graduate with distinction because of this project and my work in the Honors College. This

project was professionally important to me for two different reasons. Firstly, coming into college, I had no idea what I wanted to do after college, let alone high school. I knew that I enjoyed biology, and that my genetics units were always my favorites in my biology classes. But beyond that, I had no clue what to do with my career. However, through this project, I have learned that I actually like genetic research, and while I'm not hugely thrilled with presentations and papers, I also recognize that if I want to do genetics research, it includes the presentations and papers. Secondly, if I want to continue my education, which I do, or even get a job related to any kind of research, I need to have experience. This project has taken place over two years and has been a tedious process. But it is also wonderful practice for any upcoming research I do in my future. I had to learn a new technique, work around a few obstacles, run and analyze data, make conclusions, and share my data.

References

Davies, K. M., Schwinn, K. E., Deroles, S. C., Manson, D. G., Lewis, D. H., Bloor, S. J., & Bradley, J. M. (2003). Enhancing anthocyanin production by altering competition for substrate between flavonol synthase and dihydroflavonol 4-reductase. *Euphytica*, *131*, 259–268.

Davis, S. L., & Dudle, D. A. (2016). Time Course Pinkness Index Data

Davis, S. L., Dudle, D. A., Nawrocki, J. R., Freestone, L. M., Konieczny, P., Tobin, M. B., & Britton, M. M. (2014). Sexual Dimorphism of Staminate- and Pistillate-Phase Flowers of Saponaria officinalis (Bouncing Bet) Affects Pollinator Behavior and Seed Set. *PLoS ONE*, *9*(4). doi: 10.1371/journal.pone.0093615

Davis, S. L., & Turner-Jones, L. (2008). Potential for mixed mating in the protandrous perennial Saponaria officinalis (Caryophyllaceae). *Plant Species Biology*,*23*(3), 183-191. doi:10.1111/j.1442-1984.2008.00219.x

Delph, L. F., & Lively, C. M. (1989). The Evolution of Floral Color Change: Pollinator Attraction Versus Physiological Constraints in Fuchsia excorticata. *Evolution*, *43*(6), 1252-1262. doi: 10.2307/2409360

Dominguez, R., & Holmes, K. C. (2011). Actin structure and function. *Ann.Rev. Biophya. 40,* 169-186. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3130349/

Freestone, L. M., Konieczny, P., Tobin, M. B., & Britton, M. M. (2014). Sexual Dimorphism of Staminate- and Pistillate-Phase Flowers of Saponaria officinalis (Bouncing Bet) Affects Pollinator Behavior and Seed Set. *PLoS ONE,9*(4), 1-14. doi:10.1371/journal.pone.0102865

Farzad, M., Griesbach, R., Hammond, J., Weiss, M. R., & Elmendorf, H. G. (2003). Differential expression of three key anthocyanin biosynthetic genes in a color-changing flower, Viola

cornuta cv. Yesterday, Today and Tomorrow. *Plant Science,165*(6), 1333-1342. doi:10.1016/j.plantsci.2003.08.001

Glover, B. J., & Martin, C. (2012, March 5). Anthocyanins. *Current Biology,22,* R147-R150. Retrieved from https://www.sciencedirect.com/science/article/pii/S096098221200022X

Jabbari, S. G., Davis, S. L., & Carter, E. J. (2012). Interaction between floral color change and gender transition in the protandrous weed Saponaria officinalis. *Plant Species Biology,28*(1), 21-30. doi:10.1111/j.1442-1984.2011.00352.x

Kang, J.-H., McRoberts, J., Shi, F., Moreno, J. E., Jones, A. D., & Howe, G. A. (2014, March 1). The Flavonoid Biosynthetic Enzyme Chalcone Isomerase Modulates Terpenoid Production in Glandular Trichomes of Tomato. Retrieved from http://www.plantphysiol.org/content/164/3/1161

Kawabata, S., Li, Y., Saito, T., & Zhou, B. (2009). Identification of differentially expressed genes during flower opening by suppression subtractive hybridization and cDNA microarray analysis in Eustoma grandiflorum. *Scientia Horticulturae*, *122*(1), 129–133. doi: 10.1016/j.scienta.2009.03.011

Lloyd, D. G., & Webb, C. J. (1986). The avoidance of interference between the presentation of pollen and stigmas in angiosperms I. Dichogamy. *New Zealand Journal of Botany,24*(1), 135-162. doi:10.1080/0028825x.1986.10409725 13

Makino, T. T., & Ohashi, K. (2016). Honest signals to maintain a long-lasting relationship: Floral colour change prevents plant-level avoidance by experienced pollinators. *Functional Ecology,31*(4), 831-837. doi:10.1111/1365-2435.12802

Nawrocki, J. (2013). An analysis of the relationship between floral color, pollinator behavior, and fitness in the protandrous weed *Saponaria officinalis* (University of Indianapolis, Unpublished Honors Thesis)

Samanta, A., Das, G., & Das, S. K. (2011). Roles of Flavonoids In Plants. *Int J Pharm Sci Tech*,

6(1), 12–35.

Weiss, M. R. (1995). Floral Color Change: A Widespread Functional Convergence. *American Journal of Botany,82*(2), 167. doi:10.2307/2445525

Willson, M. F. (1994). Sexual Selection in Plants: Perspective and Overview. *The American Naturalist*, *144*, S13-S39. doi: 10.1086/285651

Appendix

CITI PROGRAM

Completion Date 04-Oct-2018
Expiration Date 03-Oct-2023
Record ID 28965036

This is to certify that:

Taylor York

Has completed the following CITI Program course:

Responsible Conduct of Research (RCR) (Curriculum Group)
Group 8: Responsible Conduct of Research (Course Learner Group)
1 - Basic Course (Stage)

Under requirements set by:

University of Indianapolis

CITI
Collaborative Institutional Training Initiative

Verify at www.citiprogram.org/verify/?w85053dee-1fce-47fe-983e-5e9579018bf1-28965036

Appendix II

An ANOVA statistical analysis describing the effect of location (grown in sun, grown in shade, or grown in the nature park) on pinkness of late pistillate phase flowers.

Dependent Variable: Pinkness					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.019[a]	2	.010	1.876	.164
Intercept	1.321	1	1.321	258.806	.000
Exposure	.019	2	.010	1.876	.164
Error	.245	48	.005		
Total	2.187	51			
Corrected Total	.264	50			

a. R Squared = .072 (Adjusted R Squared = .034)

Appendix III

Pearson Correlation Coefficient of Pinkness describing that DFR expression is not correlated at all with how pink late phase pistillate flowers get.

	PI	expression	
PI	Pearson Correlation	1	-.050
	Sig. (1-tailed)		.373
	N	51	45
expression	Pearson Correlation	-.050	1
	Sig. (1-tailed)	.373	
	N	45	47

Appendix IV

ANOVA describing the effect of the life stage of the *Saponaria officinalis* on the amount of DFR expression.

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Corrected Model	2894.332[a]	5	578.866	4.217	.003
Intercept	13583.461	1	13583.461	98.959	.000
Stage	2894.332	5	578.866	4.217	.003
Error	5765.060	42	137.263		
Total	22055.889	48			
Corrected Total	8659.391	47			

Appendix V

Tukey HSD and Tamhane analysis. Each stage is broken down and compared to each of the other four individual stages to look for significant differences between the two stages. Significant differences are those whose Sig. column is less than 0.05.

						95% Confidence Interval	
	(I) Stage	(J) Stage	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Tukey HSD	A	B	20.5513*	5.69293	.010	3.5565	37.5461
		C	6.8151	5.85797	.851	-10.6724	24.3026
		D	13.1052	5.85797	.243	-4.3823	30.5927
		E	-.8074	5.85797	1.000	-18.2949	16.6801
		F	12.8417	6.06358	.298	-5.2596	30.9430
	B	A	-20.5513*	5.69293	.010	-37.5461	-3.5565
		C	-13.7362	5.69293	.175	-30.7310	3.2586
		D	-7.4461	5.69293	.779	-24.4409	9.5487
		E	-21.3587*	5.69293	.007	-38.3535	-4.3639
		F	-7.7096	5.90428	.780	-25.3354	9.9161
	C	A	-6.8151	5.85797	.851	-24.3026	10.6724
		B	13.7362	5.69293	.175	-3.2586	30.7310
		D	6.2901	5.85797	.889	-11.1974	23.7776
		E	-7.6225	5.85797	.783	-25.1100	9.8650
		F	6.0266	6.06358	.917	-12.0747	24.1279
	D	A	-13.1052	5.85797	.243	-30.5927	4.3823
		B	7.4461	5.69293	.779	-9.5487	24.4409
		C	-6.2901	5.85797	.889	-23.7776	11.1974
		E	-13.9126	5.85797	.188	-31.4001	3.5749

		F	-.2635	6.06358	1.000	-18.3648	17.8378
	E	A	.8074	5.85797	1.000	-16.6801	18.2949
		B	21.3587*	5.69293	.007	4.3639	38.3535
		C	7.6225	5.85797	.783	-9.8650	25.1100
		D	13.9126	5.85797	.188	-3.5749	31.4001
		F	13.6491	6.06358	.237	-4.4522	31.7504
	F	A	-12.8417	6.06358	.298	-30.9430	5.2596
		B	7.7096	5.90428	.780	-9.9161	25.3354
		C	-6.0266	6.06358	.917	-24.1279	12.0747
		D	.2635	6.06358	1.000	-17.8378	18.3648
		E	-13.6491	6.06358	.237	-31.7504	4.4522
Tamhane	A	B	20.5513	6.16505	.148	-4.9681	46.0708
		C	6.8151	6.56452	.997	-18.4378	32.0680
		D	13.1052	6.14990	.645	-12.4478	38.6582
		E	-.8074	9.34745	1.000	-33.8948	32.2800
		F	12.8417	6.38982	.694	-12.4509	38.1344
	B	A	-20.5513	6.16505	.148	-46.0708	4.9681
		C	-13.7362*	3.05387	.014	-25.1071	-2.3654
		D	-7.4461*	2.01357	.032	-14.4379	-.4543
		E	-21.3587	7.32174	.268	-52.0580	9.3406
		F	-7.7096	2.65762	.199	-17.6372	2.2180
	C	A	-6.8151	6.56452	.997	-32.0680	18.4378
		B	13.7362*	3.05387	.014	2.3654	25.1071
		D	6.2901	3.02316	.622	-5.0780	17.6583
		E	-7.6225	7.66114	.998	-37.8829	22.6379

	F	6.0266	3.48540	.819	-6.4532	18.5064
D	A	-13.1052	6.14990	.645	-38.6582	12.4478
	B	7.4461*	2.01357	.032	.4543	14.4379
	C	-6.2901	3.02316	.622	-17.6583	5.0780 E
	E	-13.9126	7.30899	.779	-44.6441	16.8189
	F	-.2635	2.62228	1.000	-10.1869	9.6600
E	A	.8074	9.34745	1.000	-32.2800	33.8948
	B	21.3587	7.32174	.268	-9.3406	52.0580
	C	7.6225	7.66114	.998	-22.6379	37.8829
	D	13.9126	7.30899	.779	-16.8189	44.6441
	F	13.6491	7.51198	.812	-16.7472	44.0454
F	A	-12.8417	6.38982	.694	-38.1344	12.4509
	B	7.7096	2.65762	.199	-2.2180	17.6372
	C	-6.0266	3.48540	.819	-18.5064	6.4532
	D	.2635	2.62228	1.000	-9.6600	10.1869
	E	-13.6491	7.51198	.812	-44.0454	16.7472

The error term is Mean Square (Error) = 137.263.

*. The mean difference is significant at the 0.05 level.

Appendix VI

ANOVA describing the effect of the life stage of the *Saponaria officinalis* on the amount of DFR expression. However, this test does not include the Bud Stage

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2234.543[a]	4	558.636	5.200	.002
Intercept	8607.829	1	8607.829	80.124	.000
Stage	2234.543	4	558.636	5.200	.002
Error	3652.660	34	107.431		
Total	14582.275	39			
Corrected Total	5887.203	38			

a. R Squared = .380 (Adjusted R Squared = .307)

Appendix VII

Tukey HSD and Tamhane analysis. Each stage is broken down and compared to each of the other four individual stages to look for significant differences between the two stages. Significant differences are those whose Sig. column is less than 0.05. This data analysis is without the Bud Stage.

						95% Confidence Interval	
	(I) Stage	(J) Stage	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Tukey HSD	B	C	-14.9306*	5.18245	.050	-29.8537	-.0075
		D	-8.6405	5.18245	.467	-23.5636	6.2826
		E	-22.5531*	5.18245	.001	-37.4762	-7.6300
		F	-8.9040	5.36435	.471	-24.3509	6.5429
	C	B	14.9306*	5.18245	.050	.0075	29.8537
		D	6.2901	5.18245	.744	-8.6330	21.2132
		E	-7.6225	5.18245	.588	-22.5456	7.3006
		F	6.0266	5.36435	.793	-9.4203	21.4735
	D	B	8.6405	5.18245	.467	-6.2826	23.5636
		C	-6.2901	5.18245	.744	-21.2132	8.6330
		E	-13.9126	5.18245	.077	-28.8357	1.0105
		F	-.2635	5.36435	1.000	-15.7104	15.1834
	E	B	22.5531*	5.18245	.001	7.6300	37.4762
		C	7.6225	5.18245	.588	-7.3006	22.5456
		D	13.9126	5.18245	.077	-1.0105	28.8357
		F	13.6491	5.36435	.104	-1.7978	29.0960

	F	B	8.9040	5.36435	.471	-6.5429	24.3509
		C	-6.0266	5.36435	.793	-21.4735	9.4203
		D	.2635	5.36435	1.000	-15.1834	15.7104
		E	-13.6491	5.36435	.104	-29.0960	1.7978
Tamhane	B	C	-14.9306*	2.84569	.006	-25.4939	-4.3673
		D	-8.6405*	1.68115	.002	-14.3509	-2.9300
		E	-22.5531	7.23738	.151	-51.1953	6.0891
		F	-8.9040	2.41552	.058	-18.0702	.2622
	C	B	14.9306*	2.84569	.006	4.3673	25.4939
		D	6.2901	3.02316	.477	-4.3597	16.9400
		E	-7.6225	7.66114	.986	-35.8501	20.6051
		F	6.0266	3.48540	.680	-5.7137	17.7670
	D	B	8.6405*	1.68115	.002	2.9300	14.3509
		C	-6.2901	3.02316	.477	-16.9400	4.3597
		E	-13.9126	7.30899	.635	-42.4220	14.5967
		F	-.2635	2.62228	1.000	-9.5549	9.0279
	E	B	22.5531	7.23738	.151	-6.0891	51.1953
		C	7.6225	7.66114	.986	-20.6051	35.8501
		D	13.9126	7.30899	.635	-14.5967	42.4220
		F	13.6491	7.51198	.671	-14.6452	41.9434
	F	B	8.9040	2.41552	.058	-.2622	18.0702
		C	-6.0266	3.48540	.680	-17.7670	5.7137

	D	.2635	2.62228	1.000	-9.0279	9.5549
	E	-13.6491	7.51198	.671	-41.9434	14.6452

Based on observed means.
The error term is Mean Square (Error) = 107.431.

*. The mean difference is significant at the 0.05 level.

Appendix VIII

A Tukey HSD. This table is used to group the stages into subgroups. This is done by grouping together the stages that did not have significant differences from one another. With this data, there are only two subsets. Subset one has Early Staminate, Late Staminate, Early Pistillate, and Late Pistillate, all which do not have a significant difference between their expression of DFR. Subset two has Late Staminate, Early Pistillate, Mid Pistillate, and Late Pistillate, all which do not have a significant difference between their expression of DFR. This data was analyzed without the Bud Stage.

			Subset	
	Stage	N	1	2
Tukey HSD[a,b,c]	B	8	3.8720	
	D	8	12.5125	12.5125
	F	7	12.7760	12.7760
	C	8	18.8026	18.8026
	E	8		26.4251
	Sig.		.055	.084

The error term is Mean Square (Error) = 107.431.

a. Uses Harmonic Mean Sample Size = 7.778.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = 0.05.