

**Effects of quantity and distribution of pollen on fertilization in the
gynodioecious species *Geranium richardsonii***

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Many plants in nature fail to set the full seed set that they are anatomically capable of producing. This may be due to factors such as pollen limitation. The effects of stigma lobe removal, and natural pollination among stigma lobes, were tested in two populations of the gynodioecious plant *Geranium richardsonii*, which has the potential to set 5 seeds. The amount of stigma lobes removed limited seed set, and the majority of naturally pollinated plants received pollen on all 5 stigma lobes. It appears that there is a 1:1 anatomical relationship between stigma lobe and ovule, and this consequently affects the seed set- the less stigma lobes that are pollinated, the lower the seed set.

Introduction:

Gynodioecy is a mode of sexual expression where two different reproductive morphologies are expressed within the same population. Hermaphroditic morphs produce male and female reproductive organs (stamens and pistils with their associated structures), and female morphs are male-sterile, producing only the female sexual organs. Hermaphrodites tend to be larger and more attractive to pollinators in their floral display, while females are generally smaller and less showy (Lloyd 1975, Delph 1996, Williams et. al. 2000, Shykoff et. al. 2003). Because the two morphs have different capacities for reproduction, gynodioecy creates a situation where the stable existence of the co-occurring morphs under natural selection comes into question (Darwin 1877).

As female plants produce only the female sexual organs, they have only one pathway for reproduction- being fertilized by a different plant (an out-crossed hermaphroditic pollen source) and producing seeds. Hermaphrodites, on the other hand, have the ability to pass their genes on through three different mechanisms: the female pathway of having their ovules fertilized by another plant's pollen, and the male pathways by either fertilizing a different plant with its pollen or self-fertilization of the ovules with the plant's own pollen (geitonogamy or autogamy) - thereby reproducing via both male and female function. Thus, as Darwin hypothesized back in 1877, female plants need to compensate for their lack of male function by increased seed production and/or production of higher quality offspring in order to be maintained within the population (Lewis 1941, Lloyd 1975, Charlesworth & Charlesworth 1978). As females put less energy into creating large floral displays to attract pollinators, or to create the male reproductive organs, they may benefit from having extra resources to allot to other functions such as seed number and/or quality (Darwin 1877, Delph 1996, Shykoff et. al. 2003). Female morphotypes may also benefit from their inability to self fertilize, thus eliminating the major source of inbreeding depression in plant populations, and also eliminating a possible source of reduced seed set and lower quality offspring (Darwin 1877, Lloyd 1975, Charlesworth and Charlesworth 1978).

Despite the genetic drawbacks to self-fertilization, the opportunity for autogamy (pollination from the same flower) and geitonogamy (pollination from a different flower from the

same plant) may have persisted evolutionarily for a good reason. Pollen limitation is inadequate amounts of pollen availability to fertilize all ovules in plant populations. This can be caused by a decrease in visits by pollinators, less pollen being delivered per visit, or a reduced quality of pollen being delivered as a result of self-crossed or incompatible pollen (Ashman et. al. 2004). Chronic pollen-limitation can lead to selection for reproductive or life history traits such as self-fertilization to combat these problems, or if selection does not occur quickly enough it can lead to decreased populations or even local extinctions (Ashman et. al. 2004).

In situations where pollinator visits are limited (pollen limitation), hermaphroditic plants may have the ability to self pollinate, thereby ensuring that their genes get passed on (reproductive assurance) (Ashman et. al. 2004, Goodwillie et. al. 2005). If female plants, on the other hand, fail to be pollinated during their reproductive season, their genetic lineage is essentially halted. Under conditions of pollen limitation, females have a limited ability to pass on their genes. In this situation, one would expect there to be a gradual elimination by natural selection of female morphotypes within gynodioecious populations. Thus, the question persists, why do we still see them?

In reality, female/hermaphroditic ratios seem to have a rather cyclic and dynamic population sequence, where one morph increases in population percentage under certain conditions, and the other increases under different conditions (McCauley, D.E., and T.B. Brock 1998). As stated above, females have a reproductive advantage in times of excess pollen availability as their major difficulty is pollen limitation. Thus female populations should see an increase in numbers under such conditions as their fitness would be greater than hermaphroditic fitness (where self-fertilization, i.e. inbreeding, would still hamper their genetic success). However, as female populations increase, the pollen load available decreases, being that hermaphrodites are the only pollen source in the population (Williams et. al. 2000). If pollen loads decrease, females will fail to be fertilized, hermaphrodites may then benefit from self pollination, and thus selection will favor hermaphrodites. Therefore pollen limitation may be the factor that determines female fitness and population sex ratios.

Plants typically produce more flowers and ovules than they successfully mature into fruit and seeds. Less than maximum seed set may result from either pollen or resource limitation (Ashman et. al. 2004). If a plant's stigma receives an inadequate amount of pollen grains it may fail to maximize ovule fertilization, reducing seed set. Previous studies have looked into whether the addition of supplemental pollen to a female phase plant will increase seed set. Williams (2005- unpublished data) saw that the addition of supplemental pollen to *G. richardsonii* during the female phase of the flower still failed to produce consistent full seed sets (Figure 1).

Thus, for this project we examined the effects of various amounts and distributions of pollen on seed set on the stigma lobes of both female and hermaphroditic flowers using floral manipulations. We also investigated the physical connection between individual stigma lobes and carpels and whether a stigma lobe must be pollinated to ensure full seed set.

Geranium richardsonii is a gynodioecious perennial plant that flowers from May to August and fruits from August to September. Female flowers have been noted to be smaller than hermaphrodites, exhibiting typical traits of gynodioecious plants (Williams et al. 2000). In addition, Drew (1997) (as cited in Williams et. al. 2000) found that pollinators of *G. richardsonii* preferentially visited the larger

hermaphroditic flowers over the female flowers which could possibly cause pollen limitation in females. Females may suffer pollen limitation depending on the density and frequency of the pollen-producing hermaphrodites. In areas where hermaphroditic plants are rare, the reproductive success of both genders has been found to decrease as a response to changing neighborhood sex ratios (Graff 1999). This indicates that both females and hermaphrodites are sensitive to low pollen availability, and that pollen limitation is a more important threat in populations with higher female ratios (Asikainen and Mutikainen, 2005). Many of the effects that pollen limitation has on a population are still in question. It has been noted that a large proportion of flowers that are produced by plants do not set seed (eg Lloyd 1980), yet the question remains as to whether this is a product of pollen limitation, resource limitation, or something different altogether.

In conjunction with overall pollen limitation affecting the seed set, another possible source of variation affecting seed production may be how many ovules actually become fertilized. *Geranium richardsonii* has a single stigma that splits into 5 stigma lobes, which are all chemically available to receive pollen once they bend back (Williams et al. 2000). They also have the ability to produce 5 viable seeds from the 5 functional ovules located within the ovary each flower. However, upon observation in nature, it has been found that flowers rarely produce all 5 seeds, even when hand pollinated (Rick Williams, personal communication). This raises the question as to what the effect the spatial distribution of pollen (which stigma lobes are pollinated) has in relation to which ovules are fertilized and produce seeds. As each stigma lobe appears anatomically related to a specific ovule, we hypothesize that pollen applied to a specific

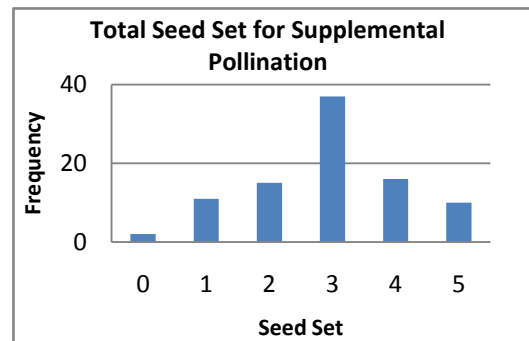


Figure 1: Average seed set for supplemental pollination plants (Williams 2005, unpublished data)

lobe will only lead to fertilization of the ovule associated with that lobe. If, however, there is no anatomical relationship between stigma lobe and ovule, we expect that stigma lobe which is pollinated to be irrelevant to which ovules are fertilized. In this case, an adequate number of pollen grains on one stigma lobe should result in full seed set.

In order to test the relationship between pollen deposition on different numbers of stigma lobes and seed production by associated ovules, we addressed the following questions and hypotheses.

Question: Is there a 1:1 anatomical relationship between stigma and ovule?

H₀₂: The location of pollen deposition on the stigma lobes of the flowers of *Geranium richardsonii* has no effect on which ovules will set seed.

H_{A2}: Pollen deposited on specific stigma lobes will correlate to the ovule associated with that ovule becoming a seed, while those not receiving pollen will fail to be fertilized.

Question: Is it necessary to pollinate each stigma lobe in order to yield the maximum number of seeds?

H₀₁: The number of stigma lobes pollinated has no effect on producing full seed set of the flowers of *Geranium richardsonii*.

H_{A1}: Full seed set will only be attained if all 5 stigma lobes are pollinated, and any less will correlate to fewer seeds being set.

We tested these hypotheses using both experimental and observational methods. Experimentally we manipulated the number of receptive stigma lobes per flower (by stigma lobe removal) and the associated seed set in those flowers. Observationally we looked at the natural distribution of pollen on stigma lobes and resulting seed set.

Methods:

Two different experiments were performed on the same set of populations, using different plants. Samples of 32 and 28 plants were taken from 2 much larger populations and were manipulated by having 0-5 of their stigma lobes removed. One site was at Gothic Townsite located near the Pump House at the junction of Gothic and Old Copper Creek Roads (PH population). In the PH population 18 hermaphroditic and 10 female plants were sampled. The other site was located at the top of the Research Meadow (RM population) of the RMBL and had 23 hermaphroditic and 16 females plants sampled. As hermaphrodites and females were not

equally distributed between the populations at different sites, more females were sampled at the Research Meadow. Both plots were fenced using DEER-X[®] protective deer fencing in order to prevent herbivory on the plants. Each plant was marked with flagging denoting plant number, and each manipulated flower was marked with a jewelry tag denoting plant number, date and treatment for reference upon return.

Before treatments (stigma removal) could be applied to hermaphroditic plants, they were emasculated while they were in their male phase in order to avoid self-pollination. This was done by removing the stamens with forceps and then removing 0-5 stigma lobes from the closed stigma. Female plants had their stigmas removed prior to their opening and receptivity. Once the stigma lobes open, they bend back and are chemically available to receive pollen and become fertilized. Thus the manipulations were done prior to this state to ensure the stigma lobes weren't pollinated before they were removed. For both morphotypes, full treatments were preformed, i.e. 0,1,2,3,4 and 5 stigma lobes were removed, when enough flowers were available.

After the unreceptive stigma lobes were removed (treatment phase) and the flowers began to set fruit, the remaining stigma lobes were collected and stained in order to count pollen grains. The stigma lobes were collected using forceps, and then transferred to slides. Slides were prepared in the laboratory before going out into the field by putting two drops of basic Fuchsin gel (Kearns and Inouye, 1993) in two different areas on the slides to allow for 2 flowers' stigma lobes to be applied per slide. The gel was re-liquefied in the field using a lighter or alcohol burner, at which point the remaining stigma lobes were applied and covered with a cover slip. These were then squashed with the back of the lighter to aid in dying and recognition of the pollen on each stigma lobe (Kearns and Inouye, 1993). After the slides were allowed to dye for 2-4 hours, pollen grains were then counted using 100x magnification on a compound microscope for each individual stigma lobe and for the total fruit.

Once the fruits began to set- about two weeks after flowering, preliminary seed set was estimated for manipulated flowers by noting how many carpels were beginning to swell. These will be compared to final seed set in case certain carpels began to swell but never set true seed. Final seed set was collected by clipping the entire developing fruit at the stem once it had turned brown, but before seeds were dispersed (approximately 2-3 days before maturity). These were placed in small coin envelopes and allowed to finish maturation and release seeds on their own. Seed set will be compared to pollen loads of individual stigmas and total flowers using Regression and ANOVA statistics.

The observational portion of the experiment was performed by looking at unmanipulated plants. Once fruits had begun to elongate (approximately 1-2 weeks after pollination), full stigma lobe sets were collected with forceps and then transferred to slides. Slides were prepared and pollen grains counted as described above.

These pollen loads were also compared to the preliminary seed set for the individual flowers that they were taken from, again by taking note of how many carpels had begun to swell. Final seed set will be taken in the same way as noted above, in order to compare actual versus estimated seed set.

Results: It should be noted that results were done on preliminary seed sets that were taken, as final seed set was not available for many plants at the time of writing.

Experimental:

PUMP HOUSE POPULATION

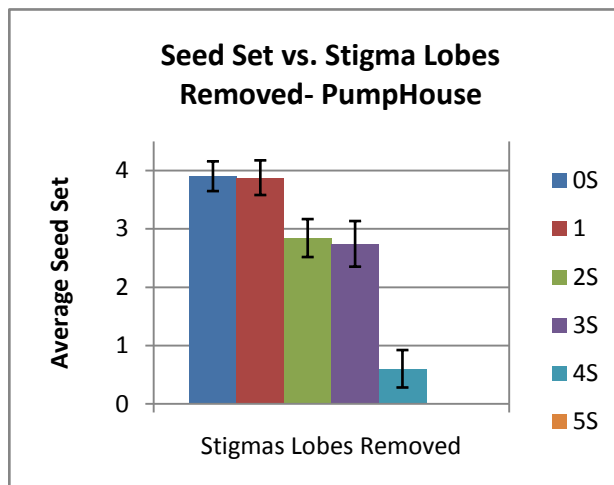


Figure 2: Average seed set vs. the number of stigmas lobes removed for the Pump House population; 0S= 0 Stigma lobes removed (5 remaining), 1S= 1 Stigma lobe removed (4 remaining), 2S= 2 Stigma lobes removed (3 remaining), 3S= 3 Stigma lobes removed (2 remaining), 4S= 4 Stigma lobes removed (1 remaining), 5S= 5 Stigma lobes removed (0 remaining).

Table 1: Sample size, means and standard errors for seed set of Pump House population of experimental stigma lobe removals.

Treatment	Count	Mean	Std. Err
0S	30	3.900	.255
1S	24	3.875	.297
2S	25	2.840	.325
3S	27	2.741	.391
4S	15	.600	.321
5S	14	0.000	0

Table 2: Statistics for Seed Set vs. Stigma Lobes removed for sample of PH population

Source	DF	SS	MS	F Ratio	P Value
# Stig	5	245.1	49.02	23.02	<0.0001
Residual	128	281.8	2.20		

There was a significant decline in seed set as the more stigma lobes were experimentally removed (Figure 1, Tables 1, 2). Intact flowers (0S) did not always set 5 seeds, while flowers with no stigmas failed to set any seed. In some cases, flowers set more seeds than expected, based on the number of stigma lobes that were removed. As seen in figure 1 there was a significant difference of average seed sets between treatments of the Pump House population with a p-value of <0.0001 (Table 2). At the 5% significance level, 2S and 3S are different from 0S and 1S, and 4S is statistically different from 0S, 1S, 2S and 3S.

Figure 3 expands on the information in Figure 2, showing the distribution of seed set for each treatment (0S-5S) individually. In the Pump House population, the modal of seed set was that predicted by the hypothesis in each treatment. However, in most treatments, some flowers

produced more seeds than can be explained by the hypothesis (those bars in blue). Thus, although seed set declines significantly among the treatments, as predicted by the hypothesis (Table 2), there was some discrepancy within each treatment, refuting a strict 1:1 relationship between number of pollinated stigma lobes and number of fertilized ovules.

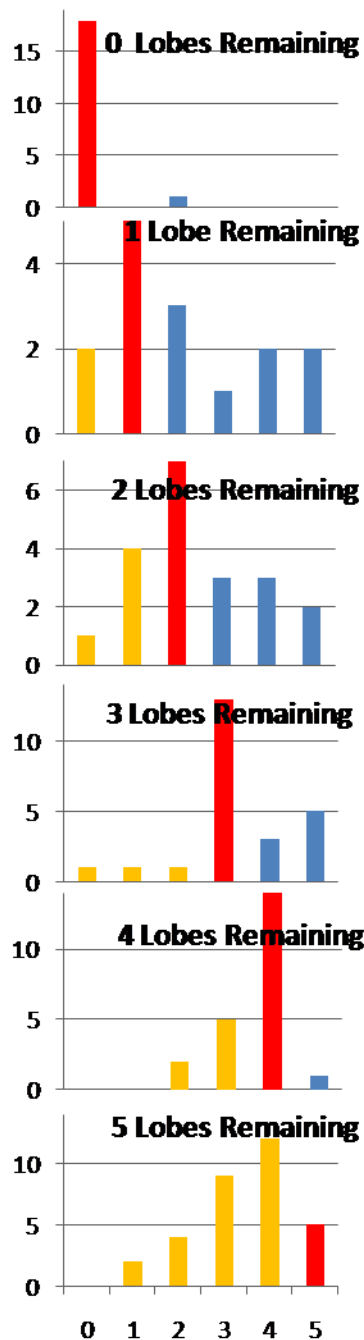


Figure 3: Average Seed set (x-axis) and frequency for Pump House population. Red denotes outcome predicted by hypothesis. Orange denotes those acceptable within the hypothesis. Blue are those unexplained by the hypothesis.

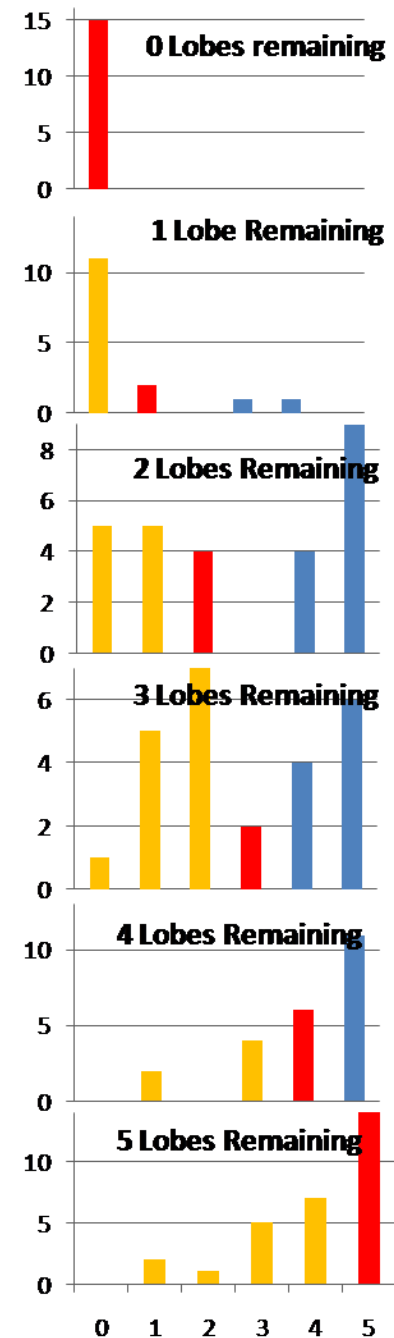


Figure 5: Average Seed set (x-axis) and frequency for Research Meadow population. Red denotes outcome predicted by hypothesis. Orange denotes those acceptable within the hypothesis. Blue are those unexplained by the hypothesis.

RESEARCH MEADOW

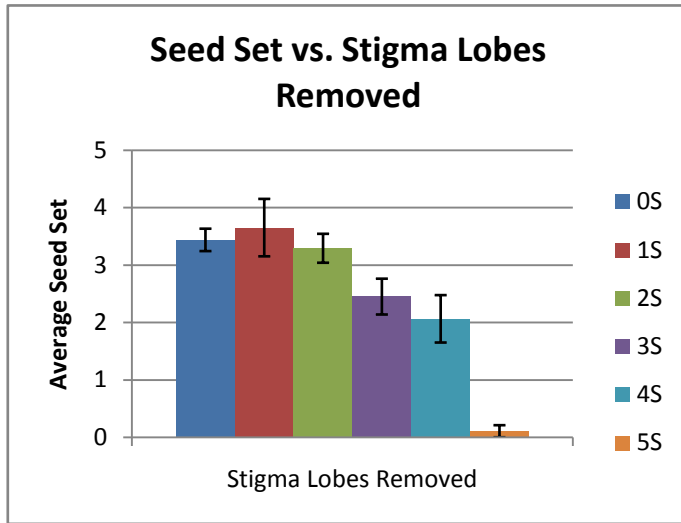


Figure 4: Average seed set vs. the number of stigma lobes removed for the Research Meadow population. Bars denote +/- standard error.

Table 3: Means and standard errors for seed sets of Research Meadow population.

Treatment	Mean	Std. Err
0S	3.438	0.195
1S	3.652	0.149
2S	3.292	0.252
3S	2.450	0.312
4S	2.063	0.413
5S	0.105	0.105

Complimentary to what was seen in the PH population, there was again a significant decline in seed set as the more stigma lobes were experimentally removed (Figure 4, Table 3). Intact flowers (0S) did not always set 5 seeds, and in fact, flowers with 1 stigma removed (1S) averaged a higher seed set than those with none removed (0S) (Figure 4). In this population, there were some flowers with no stigmas (5S) that did set seed, although the standard error was equal to the mean (Table 3). Flowers were again found to set more seeds than expected, based on the number of stigma lobes that were removed, but at higher frequencies than those found in the PH population. As can be seen in figure 4 there was a significant difference in average seed sets between treatments of the Pump House population with a p-value of <0.0001 (Table 2).

Figure 5 expands upon the information seen in Figure 4, showing the distribution of seed set for each treatment (0S-5S) individually. In the Research Meadow population, the modal of seed set was that predicted by the hypothesis in 3 of 5 treatments: 0 stigma lobes remaining (5S), 1 stigma lobe remaining (4S), and 5 stigma lobes remaining (0S). However, in most treatments, some flowers produced more seeds than can be explained by the hypothesis (those

bars in blue). Therefore, although there was again a significant decline in seed set among the treatments as predicted by the hypothesis (Table 3), there was some divergence within each treatment. These results contest a strict 1:1 relationship between number of pollinated stigma lobes and number of fertilized ovules.

Observational:

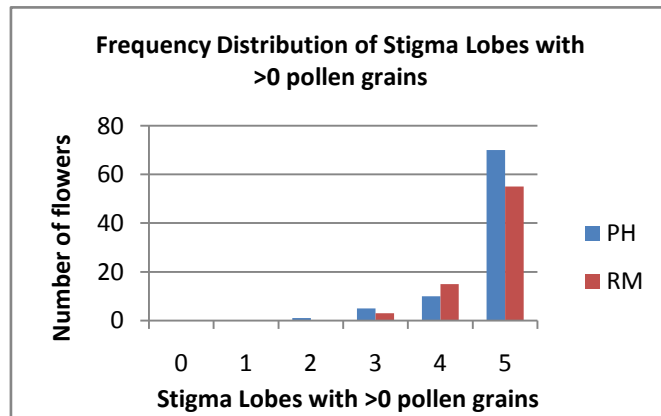


Figure 6: Relationship between the number of pollinated stigma lobes (>0 pollen grains) and seed set from intact, un-manipulated flowers in -Pump House and Research Meadow populations.

No flowers having all 5 stigma lobes pollinated set less than 4 seeds in the RM population. Although there was more variation within the PH population, no flower having all 5 stigma lobes pollinates set less than 3 seeds (Figure 7).

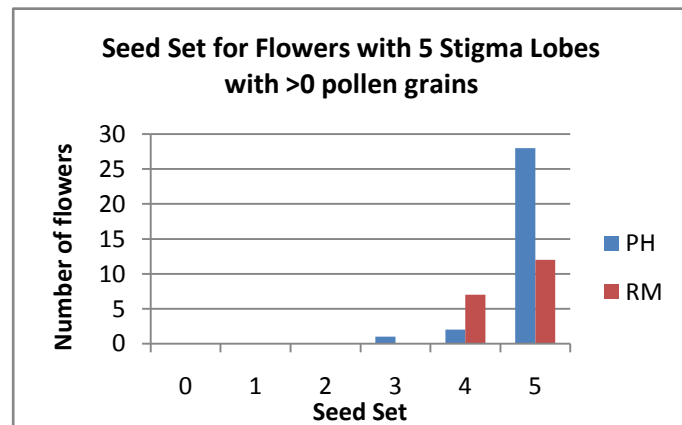


Figure 7: Seed sets for flowers with 5 stigma lobes with >0 pollen grains.

DISCUSSION AND CONCLUSIONS:

For the Pump House population, every treatment had seed sets with the highest frequency being predicted by the hypothesis (Figure 3). Essentially, when there were 3 stigma lobes remaining, 3 seeds would typically set; when there were 2 stigma lobes remaining, 2 seeds would usually set, etc. This strongly supports the hypothesis that there is a 1:1

anatomical relationship between stigma lobe and associated ovule- i.e. if a certain stigma lobe gets pollinated, that pollen tube will fertilize the ovule that is most closely related to it anatomically. More generally, it was clearly seen as the overall pattern in both populations that as more stigma lobes were removed, less seeds set (Figures 2, 4). These patterns also strongly support the 1:1 hypothesis.

Despite this, almost all treatments had some inexplicable results if the hypothesis is to be strictly correct- i.e. in many cases more seeds set than there were stigma lobes available to be pollinated. These results were found at highest frequencies within the Research Meadow population (Figure 5). Treatments for 0, 1, 3 and 5 stigma lobes remaining all had seed sets with the highest frequencies either predicted by or explained by the hypothesis. Yet, treatments for 1, 2, 3 and 4 stigma lobes remaining exhibited flowers that set more seeds than explained by the hypothesis, and treatments for 2 and 4 stigma lobes remaining had modal bars unexplained by the 1:1 hypothesis. These graphs (Figure 5) are essentially showing that even if there were only 2 stigma lobes remaining- and therefore only 2 stigma lobes that have the possibility for becoming pollinated- the flower was able to produce more than 2 seeds. This frankly contradicts the theory that a stigma lobe is directly connected to a specific ovule because if that were the case, 2 or less seeds would be setting. The same line of thinking applies for the results of the 4 stigma remaining treatment.

However, there could be underlying information found within the unexplained results. If the flowers are able to set more seeds than there are stigma lobes available to be pollinated, it appears that the pollen tubes aren't growing directly down one stigma lobe into one ovule. Perhaps instead they have the ability to grow across the style on their way down to fertilize the ovule. In this case even if only one stigma lobe receives pollen, if there are at least 5 pollen grains on the stigma lobe, the flowers has the potential to set 5 seeds. This could be tested by applying known amounts of pollen to only 1 stigma lobe and comparing that to final seed set. This could also be tested by looking closely at the growth of the pollen tube at different stages, to see in fact where it does grow.

In addition to there being higher seed set than predicted, there were even more numerous occasions where there were lower seed sets than predicted by the hypothesis. It is conceivable that instead of being a case of spatial limitation, the lack of full seed set could be due to resource limitation. Flowers may only be able to set as many seeds as the plant has energy for. As *Geranium richardsonii* flowers from the bottom of the stem upwards, a resource limitation could be relative to where the flower is on the plant, as flowers that are higher up on

the stem tend to bloom later in the season, when plants presumably have fewer resources available.

Resource limitation can also take the form of low-nutrient availability, instead of low-energy availability. Plants may have had lower seed sets than possible because they were in low-nutrient conditions. In fact, Asikainen and Mutikainen (2005) found that seed set in *Geranium sylvaticum* was in fact resource-limited, and not pollen-limited. However, as soil, water and light conditions were not assessed during our experimentation, there is no way to know for this case in particular. However, it does open the door for future studies that would yield more concrete results.

There is also the possibility that some of the unexpected results can be explained by experimental error. With this experiment in particular, the timing of manipulation was very important. With hermaphroditic plants, manipulation was more straight-forward as one could remove the anthers while the flower is in the male phase, and then simply removes stigma lobes while they are still closed up and all together. With the female plants, there is much less leeway in terms of timing. As they have no male phase, one would essentially have to catch the flower when it was first opening in order to clip stigma lobes before they entered the female phase and were receptive to pollen. In a few situations it was unclear as to whether that window had been missed or not, but the manipulations were still preformed. Thus, there is the possibility that pollen could have been on the stigma lobes before they were removed, and therefore had the ability to possibly fertilize an ovule before the experimentation fully began.

An additional source of experimental error could have been in the initial removal of unreceptive stigma lobes. In order to perform the manipulations, 0-5 stigma lobes were removed in order to ensure that they could not receive pollen (See Methods). However, when the remaining stigma lobes were collected to assess pollen loads, it was noted that a number of the full stigma lobes also included stubs of the thought-to-be-removed unreceptive stigma lobes. Additionally, some of these stubs had pollen grains on them and consequently had the potential to have pollen tube growth within them. Whether or not these stubs were chemically receptive to pollen and had the ability to fertilize the ovules remains in question, however it is a strong possibility that these could affect seed set as well.

Observationally, the results support the hypotheses as well. It was shown that the majority of flowers in the wild receive pollen on all stigma lobes. It was also shown that within those that did have pollen on all stigma lobes, they most often set 5 seeds. This, in addition to the other data, supports the hypothesis that all stigma lobes need be pollinated in order to

ensure full seed set. Thus it appears that the lack of full seed set in nature is most likely due to the deficiency of all stigma lobes receiving pollen, and thus failing to fertilize all ovules.

Regardless, it appears that the next step in this line of investigation would be to bring these experiments into a green house to control for possible pollination errors. Stigma lobes would not have to be removed in the pollinator-free environment, and by hand pollinating one would be able to account for all desired lobes receiving pollen, instead of relying on natural pollinators to get the job done.

Additionally a closer inspection into the anatomical study of the pollen tube growth from the stigma lobe would also offer valuable insight. Specifically looking into the various stages of the growth of the tube could provide more concrete evidence as to whether or not it stretches directly down to a specific ovule, or merely to some vicinity within the ovary.

Overall this study has provided valuable insight into the pollination biology of *Geranium richardsonii*. With such strong statistical evidence, it appears from the results that there is a 1:1 anatomical relationship between a stigma lobe and a specific ovule. Pollination of a stigma lobe results in the fertilization of the ovule associated with it, and the lack of pollination correlates with that ovule's failure to turn into a seed.

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