Delayed Self-Fertilization in *Triodanis perfoliata*

by

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I hereby declare I am the sole author of this thesis. It is the result of my own work and is not the outcome of work done in collaboration except for aid in conduction of pollen tube and seed set experiments, nor has it been submitted elsewhere as coursework for this or another degree.

Signed: Hetal Patel Date: 5/29/14

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Abstract

Self-fertilization is a reproductive technique that allows a plant to produce offspring even when pollinators may be scarce. Utilizing the process of delayed selfing, a species is with the benefits of both cross-pollination and self-fertilization as it has the opportunity to cross-fertilize early on in the flowers life and self-fertilize in the later stages. Research conducted on *Triodanis perfoliata*, a winter annual species, was designed to determine the timing of self-fertilization. Additionally, the study shed light on the species' ability to produce a full seed set through self-fertilization. Plants used in these experiments were grown from seeds collected from two populations: a wooded roadside in Pitt County, NC (arboretum population) and a disturbed grassy area in New Bern, Onslow County, NC (New Bern population). The plants were grown in standard potting medium containers (Stuewe and Sons, Corvallis, OR) with subirrigation and inspected every day for flower opening. Styles of various ages were collected (after flower opening) and placed in a solution. The pollen tubes that penetrated the stigma and entered into the style were counted. This helped determine that self-fertilization usually occurs towards the late stages of anthesis.

After the application of four treatments to randomly selected flowers, seed set count among the *Triodanis perfoliata* populations was compared. The first two treatments both included hand self-pollination on the first day of the female phase; however in one

treatment the style was cut to prevent selfing late in anthesis while the style in the other treatment remained uncut. In the third and fourth treatments no hand-pollination was applied but once again in one treatment the style was cut off and remained intact in the other. Comparing the unpollinated treatments with the cut and uncut style confirmed that self-pollination did occur in late anthesis. The lower seed production within the cut style treatment indicated that selfing is at least somewhat delayed. Furthermore, the treatments comparing the cut and uncut style indicated that the removal of the style only prevented delayed selfing and had no additional effects on the experiment. The third experiment illustrated the relationship between pollination of stigma lobes and senescence of the flower. The timing between the two indicated early cross-pollination triggered senescence of the flower, preventing self-fertilization from occurring. However, if cross-pollination was not to occur early on, the flower would live a longer life, awaiting the chance to self-pollinate. Together, this research suggests that *Triodanis perfoliata* uses delayed selfing to reproduce.

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Introduction

Self-fertilization provides species with reproductive assurance in the absence of both pollinators and additional members of the species to mate with. In flowering plants, a predominant method of self-fertilization is delayed selfing, in which a flower is given the chance to both cross-fertilize and self-fertilize (Fan & Li, 2012). In this form of reproduction the flower is able to cross-fertilize early on in the flowers life and has the chance to self-fertilize later on if cross-fertilization is not achieved.

Delayed selfing ensures that a flowering species has the opportunity to acquire the benefits that cross-fertilization offers. A negative consequence of self-fertilization as the sole form of reproduction is inbreeding depression, reduced fitness among a population due to breeding between closely related individuals, which may outweigh any benefits the reproductive process has to offer (Charlesworth & Charlesworth, 1987). Inbreeding depression is the result of genetically similar parents passing on and expressing recessively deleterious alleles in their offspring. Expression of these recessive traits can characterize highly unfit individuals. When given the chance to self-fertilize and cross-fertilize, a species thrives from advantages offered by both reproductive methods. In combination of both methods, the risk of inbreeding depression reduces for the species.

Of the species known to use delayed selfing, several mechanisms have been reported (Fan & Li, 2012, Table 1). One commonly studied mechanism deals with various forms of style curvature. In *Kosteletzkya virginica*, the style has been shown to curve downwards towards the flower's anthers during anthesis. If pollination does not occur, this method allows for the flower's stigma lobes to come into contact with pollen on the anthers

late in anthesis (Ruan et al., 2009). Another studied delayed selfing mechanism is known as corolla dragging. The species *Incarvillea sinesis* has been found to exhibit a form of this mechanism. When cross-pollination fails to occur, self-fertilization can occur by the wind abscising the corolla and causing it to drag the adherent epipetalous stamens. This causes the anthers to come into contact with the stigma (during late stages of the flowers life) and allow for self-fertilization (Qu et al., 2007). These mechanisms, along with others, are hypothesized to be present to allow outcrossing to occur but ensure some offspring production in the absence of pollinators.

Table 1. Mechanisms of delayed selfing reported in the literature

Mechanism	Species Reportedly Utilizing Mechanism
Poorly developed clinandrium pushes pollen grains towards stigma during senescence	*Epipactis helleborine* (Orchidaceae) (Suetsugu, 2013)
Style curvature	Kosteletzkya virginica (Malvaceae) (Ruan et al., 2009)
	Zygophyllum mcaropterum (Zygophyllaceae) (Mamut, Li, & Tan, 2014)
Corolla dragging	*Ruellia succelenta* (Acanthaceae) (Geiger, Pratt, & Koptur, 2010)
	Incarvillea sinensis (Qu et al., 2007)
Transient Self-incompatibility-delayed receptivity of the stigma	*Leptosiphon jepsonii* (Polemoniaceae) (Goodwillie, Partis, & West, 2004)
	Arabidopsis thaliana (Liu, Sherman-Broyles, Nasrallah, & Nasrallah, 2007)
	Sebaea aurea (Kissling & Barrett, 2013)
	Collinsia Verna (Kalisz, et al., 2013)
Stigmatic Fluid Globule	*Roscoea debilis* (Zingiberaceae) (Fan & Li, 2012)

Triodanis perfoliata is commonly found along roadsides and disturbed areas throughout North America. The two subspecies of *T. perfoliata* -- *T. perfoliata* ssp. *perfoliata and T. perfoliata* ssp. *biflora* -- both contain chasmogamous (open) and cleistogamous (closed) flowers. This protandrous species has a male stage for the initial 1-3 days of each flower's life, followed by a female stage that lasts until flower senescence. We hypothesized that self-fertilization might occur in this species as the stigma lobes curl backwards during the female stage. As the receptive areas of the lobes come into contact with the pollen on the style (initially deposited by anthers before anthesis), self-fertilization could occur. Because this process is thought to only occur in later stages of the flowers life, to provide an opportunity for cross-fertilization, the species is hypothesized to use delayed selfing.

Experiments were conducted to determine the reproductive habits of *Triodanis perfoliata* ssp. *perfoliata*, hereafter referred to as *T. perfoliata*. The mechanism and timing of *T. perfoliata* reproduction was analyzed through three experiments dealing with the following: pollen tube growth, seed set, and flower senescence. The pollen tube growth experiment was conducted to study when self-fertilization occurred within the flowers. After anthesis, hand-pollinated flowers of varying ages were collected. The number of pollen tubes penetrating the stigma was counted to determine how self-fertilization varied over time. The seed set experiment was aimed towards gaining a better understanding of the extent of autonomous selfing (self-fertilization with the aid of a pollinator) alone and confirm that self-fertilization was only used in later stages of the flower's life. Four treatments, in which hand-pollination and style cutting varied, were applied. The cut and uncut style treatments were implemented to determine how seed set differed among

flowers that could not self-pollinate in late stages. Cut style flowers did not have the opportunity to fertilize ovules from late-deposited pollen. Seed sets were analyzed for variation between flowers that could and could not use delayed selfing to ensure seed production. The final flower senescence experiment helped define the relationship between pollination and senescence. Four treatments of control, varying hand-pollination amounts, and emasculation were applied and compared for their impact on the number of days between the female stage of the flower and its senescence. Two treatments, full and half hand-pollination of stigma lobes, were applied to determine if pollination could trigger senescence, thus preventing delayed self-fertilization from occurring. These results were compared to those of the control group in which no hand-pollination was applied as this group would be expected to senesce later. A shorter amount of time found in the pollination treatments than in the control would indicate that flower senescence is triggered after cross-pollination, preventing the need for delayed self-fertilization. The emasculated stage was expected to have the longest time period between female stage and senescence as no pollen could trigger its early senescence. These experiments not only tested whether or not the species used delayed selfing to reproduce, but also the degree to which self-fertilization alone proved to be successful for seed set.

Materials and Methods

Study species. *Triodanis perfoliata* produces chasmogamous flowers in lower axils and a few cleistogamous flowers in upper axils. Chasmogamous flowers (hereafter, referred to simply as "flowers") are 1-1.5 cm in diameter with five blue to violet petals. Before anthesis, anthers held tightly around the stigma dehisce and deposit pollen onto the outer stigma surface. When flowers first open the stigma lobes remain closed, and pollen is presented for dispersal by pollinators for 1-2 days, constituting the male phase. Stigma lobes then open to begin the female phase, revealing the receptive surface to which pollen is deposited by visiting insects. Stigma lobes become progressively curled backward toward the petals each day of anthesis, such that the receptive surface eventually contacts the outer stigma surface on which self-pollen is presented. Small bees, wasps, and beetles are common visitors to the flowers of *T. perfoliata* (Gara & Muenchow, 1990). Fertilized flowers produce capsules, which dehisce through small pores when mature, releasing seeds.

Pollen tube growth experiment. To study the timing of self-fertilization, we quantified self-pollen tube growth in flowers throughout anthesis. Two plants from each of 12 maternal families of each population were used for the study. When plants began to form chasmogamous flowers, plants were inspected daily for flower opening. Bracts subtending the chasmogamous flower buds were marked for identification and the date of flower opening was noted during daily observations. Styles of flowers of different ages were removed and fixed in a 70:30 mixture of ethanol and acetic acid. Two flowers of each age

(1-6 days after flower opening) were collected from each plant in a haphazard order. Flower senescence was noted daily and no styles were collected more than one day after senescence. Fixed styles were cleared in 10M sodium hydroxide for 24 h and stained in 0.1% aniline blue in 33 mM potassium phosphate solution. Pollen tubes were viewed using epifluorescent microscopy. Tubes that penetrated the stigma and extended into the style were counted. Stigma lobes were also tested for receptivity on the first day of anthesis as a control group. This was done by hand-pollinating the lobes on day 1 to confirm that pollen tube growth was possible.

Seed set experiment. Seed set experiments were carried out using two additional plants of each maternal family of each population, as described above. To determine the extent of early and delayed autonomous self-fertilization, seed set was compared in flowers to which one of four treatments was applied: 1) hand self-pollinated on the first day of female phase, style cut one day later; 2) hand self-pollinated on the first day of female phase, style not cut; 3) no pollen applied, style cut on the second day of female phase; 4) no pollen applied, style not cut. Floral bracts were marked to indicate the assigned treatment before flowers opened and buds were inspected daily to determine the first day of female phase. Each treatment was applied to two replicate flowers of each experiment plant. The order of the four treatments was systematically varied across the experimental plants to avoid any confounding effects of flowering sequence on seed production. Hand self-pollinations used pollen from male phase flowers on the same plant. Styles were cut halfway between the tip and base of style. Fruits were collected when mature but before capsule dehiscence, and seeds were counted. Comparison of unpollinated/uncut and unpollinated/cut treatments

tested the primary hypothesis that self-pollination occurs late in anthesis. Significantly lower seed set from excised styles would indicate that at least some portion of self-fertilization is delayed. Comparison of unpollinated/uncut and pollinated/uncut tested whether autonomous selfing is capable of producing full seed set. The pollinated/cut treatment served as a control for unintended effects of style excision. Lack of a significant difference between pollinated/cut and pollinated/uncut treatments indicates that style excision has no effect except to prevent fertilization by delayed pollen deposition.

Flower senescence experiment. Chasmogamous flowers of *Triodanis perfoliata* were subjected to four treatments to determine the relationship between time of pollination and time of flower senescence. The four treatments were replicated on each of two flowers from each of twelve plants. The treatments included: 1) a control (no manipulation); 2) a full pollination treatment in which the entire stigma was hand-pollinated; 2) a partial-pollination treatment in which only one lobe of the stigma was hand-pollinated; 4) an emasculated treatment in which the anthers were removed before anthesis. Markings were made on the bracts underneath each flower to indicate treatment. Hand-pollination was completed on the first day of the female phase of anthesis. Flower senescence was noted each day to determine the number of days between flower opening and stigma lobes opening and the number of days between stigma lobe opening and the flower senescence. Close observation of flower wilting, fading colors, and petal shedding helped determine when flower senescence, the terminal stage of the flower's life, had occurred.

Analysis. The data collected from the three experiments were subjected to analysis of variance tests using of IBM SPSS Statistics Version 20.0 (SPSS IBM, New York, U.S.A.). Tukey test post-hoc were used to compare groups within each experiment to one another. Each experiment was analyzed individually to determine the validity of the hypothesis that *Triodanis perfoliata* reproduces through delayed self-fertilization. In the pollen tube experiment, analysis of variance tested for the effects of flower age (days) and plant on the number of pollen tubes counted. The plants were treated as a random variable and age as a fixed factor. The second experiment testing for seed set count used analysis of variance to test for the effects of treatment and plants on seed set, treating plants as a random variable and treatment as a fixed factor. Finally, the flower senescence experiment again used analysis of variance. This analysis tested for the effects of treatment and plants on the number of days between the first days of female stage to senescence. Plants were again treated as a random variable and treatments as a fixed factor.

Results

Pollen Tube Growth Experiments

Analysis of this experiment indicated how the dependent variable of self-pollen tube growth varied over the independent variable of number of days. Populations were pooled together in analysis as they were not found to be significant factors. Table 2 indicates the factors affecting pollen tube growth and the significance of each. Both factors, number of days and plants were found to significantly affect pollen tube growth, (P= 0.00 and 0.01, respectively). The effect of flower age on pollen tubes was found to vary among plants, P=0.002). The Tukey test, a form of post-hoc analysis, was conducted to show similarities and differences among the pollen tube count for flowers of different ages. Subsets represent significantly different mean pollen tube counts throughout the days of the experiment. As each subset of number of days increased, so did the number of pollen tubes (Table 3). Both populations saw a dramatic increase in pollen tube growth as flower age increased (Figure 1). The control group disproved the idea that the change in pollen tube could be attributed to lack of early stigma receptivity. Plants from both populations were hand-pollinated right after anthesis and had average numbers of of 179.75 and 194.5 pollen tubes. This showed that the stigma lobes were capable of pollen tube growth early on in the flowers life.

Table 2: Effect of flower age and plant on number of pollen tubes penetrating the stigma. Population was not included in this analysis as its effect was found to be not significant.

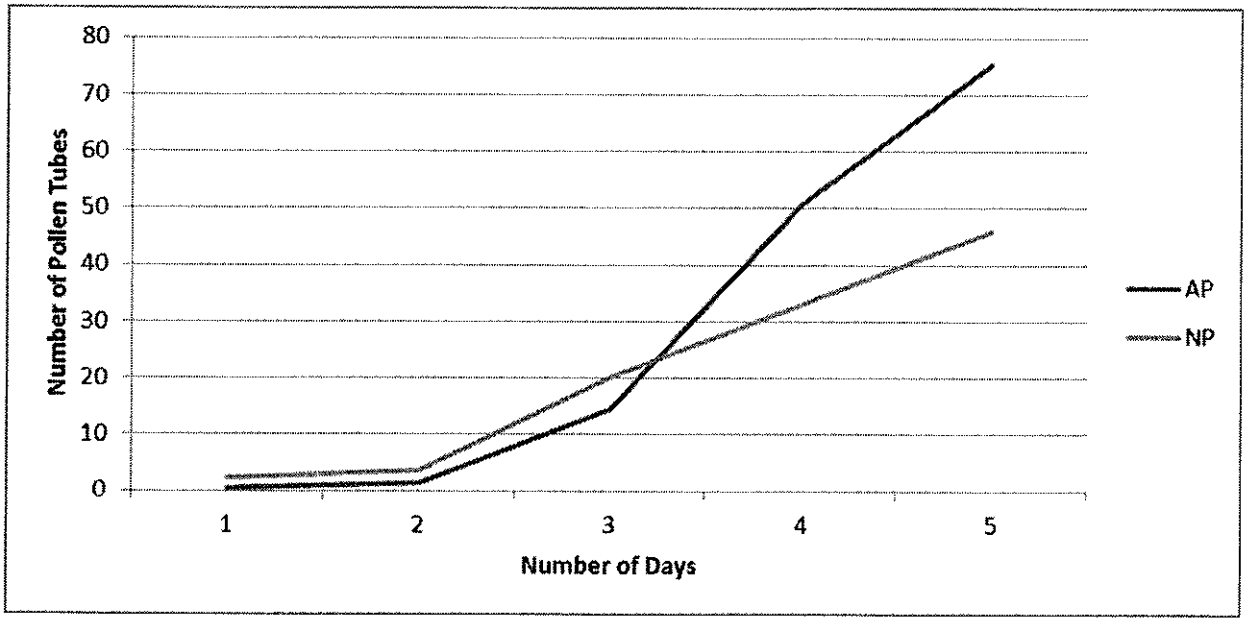
Dependent Variable: Number of self-pollen tubes

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Number of days	104868.103	6	17478.017	20.115	.000
	60138.146	69.210	868.917[b]		
Plant	32521.964	9	3613.552	3.845	.001
	51184.124	54.468	939.718[c]		
Number of days * plant	43143.552	41	1052.282	1.894	.002
	141710.999	255	555.729[d]		

Table 3: This post-hoc analysis depicts areas in which significant differences occur throughout the pollen tube experiment. The various subsets illustrate which flower ages had similar mean pollen tube growth in comparison to each other. The p-values for the significant differences among the days in each subset are shown on the bottom row.

Flower age in number of days	N	Subset			
		1	2	3	4
1	61	1.28			
2	66	2.47			
3	70	17.07	17.07		
6	5		31.00	31.00	
7	5		37.20	37.20	37.20
4	69			43.71	43.71
5	36				59.94
Sig.		.553	.255	.777	.135

Figure 1. Pollen tube count over time after anthesis among the two populations (AP and NP). Day one began when anthesis of the flower occurred. Count of the number of days passed stopped after day five as most flowers began to wilt and senesce around this time.



Seed Set Experiment

Populations were pooled together in analysis as they were not found to be significant factors. Both factors, pollination treatment and plant, were found to have a highly significant effect on seed set (Table 4). The effects of treatments and plants were found to vary significantly among plants; P=0.00 for both factors. Treatment was found to vary among plants, P=0.047. A Tukey test was used to separate the treatment into subsets based on their significant difference (Table 5). Subset 3 was the only subset which contained two treatments (pollinated/cut & pollinated/uncut), indicating there was no significant difference between them. The pollinated/cut and pollinated/uncut saw the highest mean values of the four treatments (Figure 2). The unpollinated/uncut treated plants had the lowest mean seed set value in both populations. To illustrate the varying seed set of each treatment among individual plants, a stacked bar graph is presented in Figure 3. This graph provides a visualization of similar values found in the pollinated/cut and pollinated/uncut treatments and the differing values between the pollinating/uncut and unpollinated/uncut.

Table 4: Effect of pollination treatments on seed production. Treatment was considered to be a fixed variable and plants were assigned randomly.

Dependent Variable: seed number

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	684429.791	3	228143.264	62.902	.000
	286226.460	78.917	3626.942[b]		
Plant	309540.471	26	11905.403	3.279	.000
	285154.700	78.544	3630.518[c]		
Treatment * plant	283590.882	78	3635.781	1.417	.047
	274457.667	107	2565.025[d]		

Table 5: This post-hoc analysis depicts areas in which significant differences occur throughout the experiment. The various subsets illustrate which treatments had similar mean seed set number in comparison to each other. The treatments are listed as follows: UC=unpollinated, cut; UU=unpollinated, cut; PC=pollinated, cut; PU=pollinated, uncut.

Tukey HSD

Treatment	N	Subset		
		1	2	3
UC	54	35.59		
UU	55		71.18	
PC	53			158.74
PU	53			169.08
Sig.		1.000	1.000	.716

Figure 2. Effect of pollination treatments on seed set number. The graph indicates mean values of each treatment among the two populations.

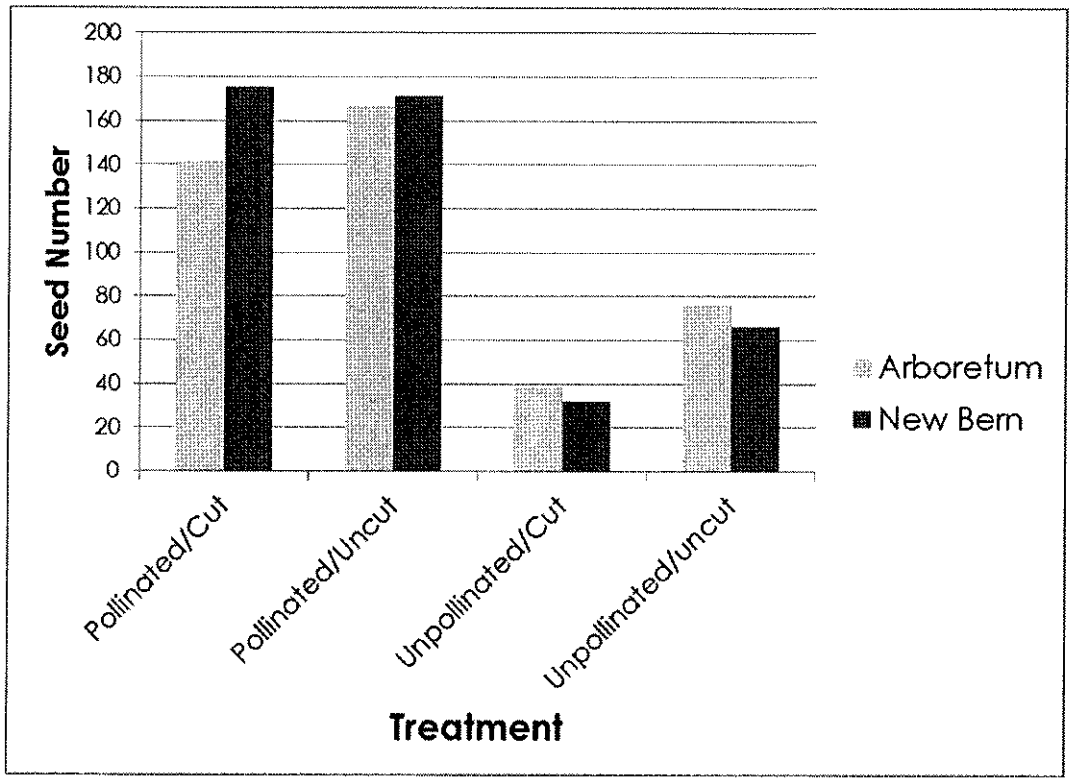
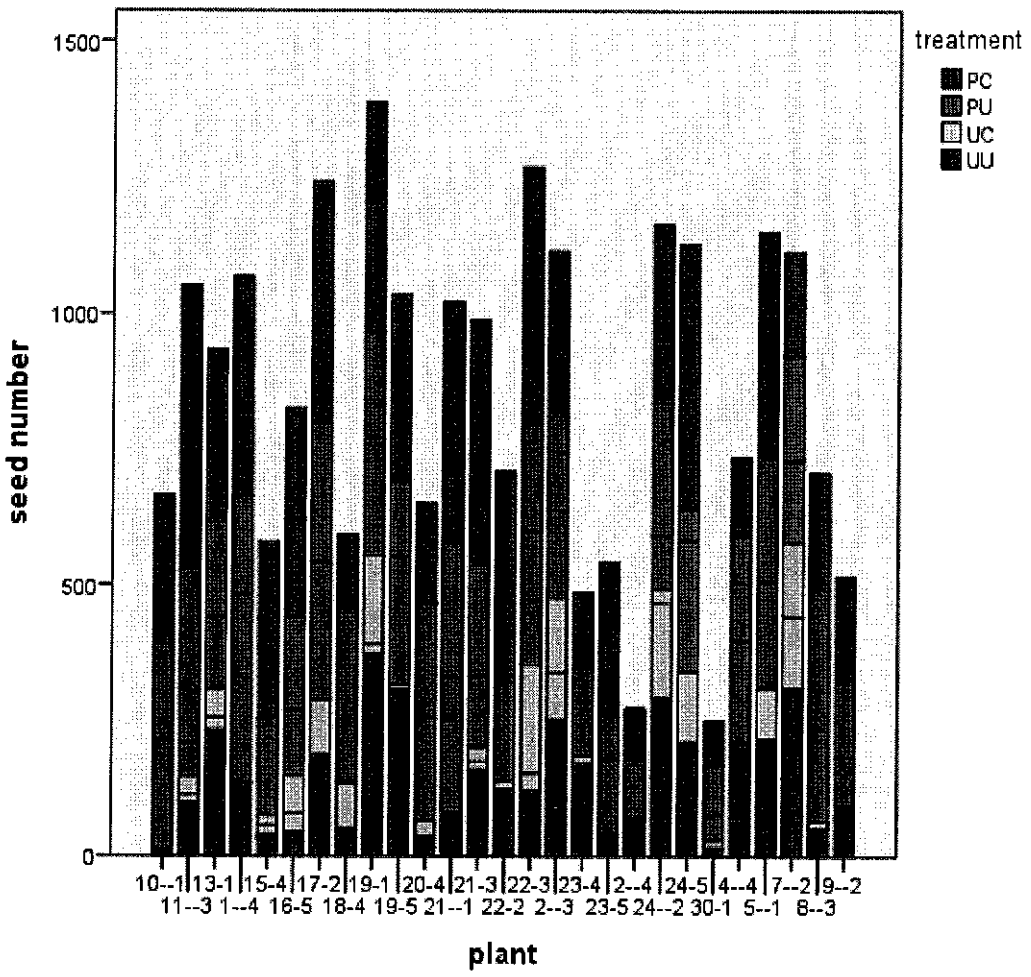


Figure 3: Variation among plants in the effect of pollination treatments on seed set. The stacked bars indicate each treatment within the same plant. Each plant was subjected to all four treatments among four individual flowers.



Flower Senescence

Four treatments were applied to determine their effect on the number of days between the start of a flower's female stage to anthesis. Both treatment and plant were found to have a significant effect on time to senescence (Table 5, P = 0.00 and 0.003, respectively). Treatment was not found to vary among plants, as indicated by P= 0.474. Once again, a Tukey test was used to segregate the treatments into subsets containing significantly different means (Table 6). The full and half pollination treatments (Subset 1) were found to be significantly different from the emasculated and control treatments (Subset 2). Figure 4 provides visualization of the average number of days required for each treatment. This graph, in addition to Table 6, shows the similarity between the full pollinated and half pollinated treatments as well as the similarity between the emasculated and control treatments. The graph also indicates the shorter amount of time between the female phase and senescence seen in the full pollinated and half pollinated treatments. The shortest time was seen in the full pollinated treatment and the longest time was seen in the emasculated treatment.

Table 6: The effect of pollination treatment on flower senescence. Treatments given to each flower were considered to be fixed variables and plant was a random factor. The final column lists p-values for variables within this experiment.

Dependent Variable: Days From Female Phase to Senescence

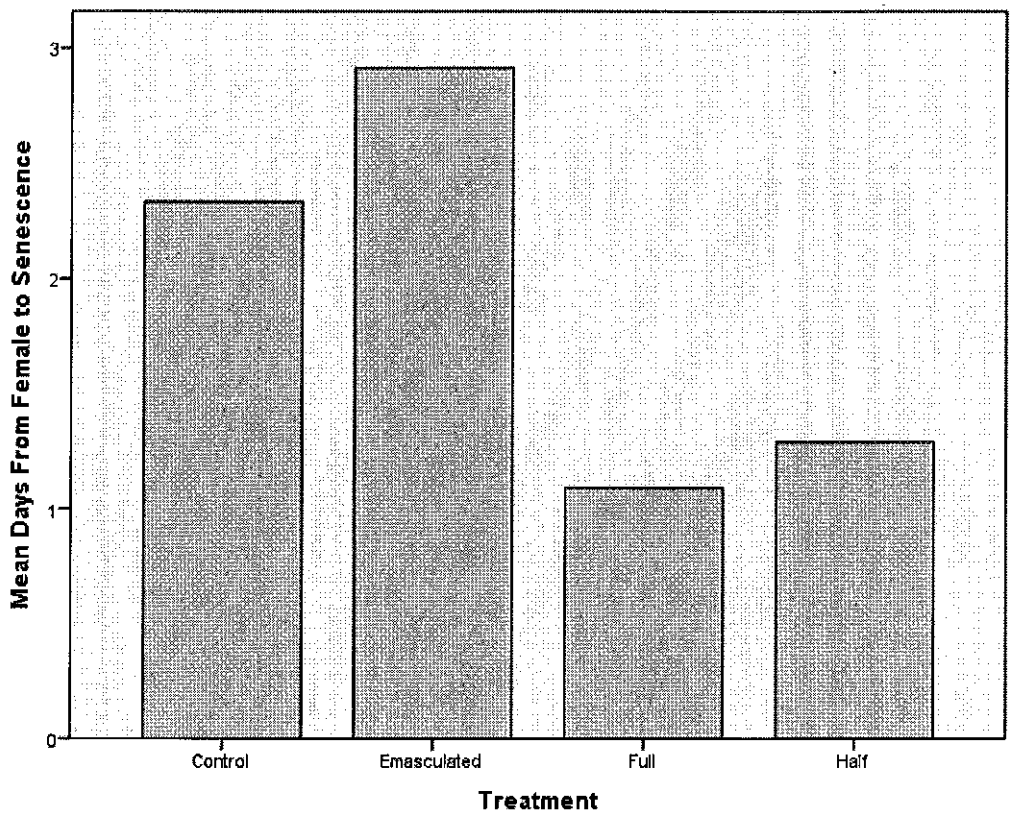
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	50.400	3	16.800	29.857	.000
	18.211	32.365	.563[b]		
Plant	20.981	11	1.907	3.390	.003
	18.346	32.608	.563[c]		
Treatment * Plant	18.007	32	.563	1.015	.474
	25.500	46	.554[d]		

Table 7: This post-hoc analysis depicts areas in which significant differences occur throughout the experiment. Each of the subsets show which treatments had similar mean days from female stage to senescence in comparison to each other. The p-values for the significant differences among treatments in a subset are shown on the bottom row.

Tukey HSD

Treatment	N	Subset	
		1	2
Full pollination	22	1.09	
Half pollination	24	1.29	
Control	24		2.33
Emasculated	23		2.91
Sig.		.795	.051

Figure 4. Effect treatments have on the number of days between female stage initiation and senescence. Full and half treatments indicate the amount of stigma lobes hand-pollinated.



Discussion

Taken together, the results from these three experiments support the hypothesis that *Triodanis perfoliata* uses delayed self-fertilization to reproduce. In the pollen tube experiment, we found that the number of self-pollen tubes present in the styles increased with floral age. Data collection began on day 1 when anthesis occurred. As days passed, the pollen tube count increased. Pollen tube counts from days 2 and 3 include tubes that most likely began forming when the flower reached female stage and began revealing its pollen receptive areas. As the female stage continued and the stigma lobes curled back, pollen tube numbers continued to increase during days 4 and 5. This suggests that stigma lobes may have come into contact with pollen produced by the flower itself. Before anthesis, anthers deposit pollen onto the style. As later stages of the flowers life arrive and the stigma lobes begin to curl backwards, the receptive areas can come into contact with the originally deposited pollen to self-fertilize. Both number of days passed and plant the flower show that both factors influenced pollen tube growth (Table 2). This experiment indicated that self-pollen tube growth increased during later stages of the flowers life, suggesting the species' use of delayed self-fertilization.

Data collected from the seed set experiment provided further understanding of whether self-fertilization was delayed and the extent to which self-fertilization alone was successful in producing full seed set. Among the four treatments, the pollinated/cut and pollinated/uncut served as a check to make sure that there were no unintended effects in the experiment from cutting the style. The comparison also tested whether cutting the style allowed time for pollen deposited at the start of female phase to reach and fertilize the

ovules. There was not a significant difference between the two treatments that were found in subset 3 (P= 0.716, Table 4). Therefore, the effect of cutting the style was only to prevent late-deposited pollen from fertilizing ovules. A comparison of unpollinated/cut and unpollinated/uncut treatments tested whether selfing occurred in early or late female phase. The separation between the two groups into subsets 1 & 2 indicates that these treatments did yield highly different results (Table 4). The higher unpollinated/uncut treatment seed set supports the idea of delayed selfing occurring because the uncut style would be able to participate in self-fertilization towards the end of the flowers life while the flowers in which the style was cut early in the female phase were not able to do so. The final test of this experiment occurred between the pollinated/uncut and unpollinated/uncut groups. This tested whether the autonomous selfing alone was enough to produce a full seed set capable of being produced through cross-fertilization. The difference between these the means for these two treatments, indicated that self-fertilization alone is substantial but is not enough to produce a full seed set, the maximum amount capable of being produced if all ovules were fertilized (Table 5). The unpollinated/uncut treatment, which allowed only autonomous selfing, yielded a significantly lower seed set than did the pollinated/uncut treatment. Figure 2 illustrates that the effect of the treatments varied among plants. Table 4 p-values confirm that there is a significant interaction between treatments and plants. This suggests that the plants differed in their responses to the treatments applied. Variation among plants in seed set values of the unpollinated/uncut treatments could be attributed to variation in the curvature of the stigma lobes, allowing the stigmas of some plants to come into contact with more pollen.

The flower senescence experiment was conducted to determine how pollination affects time between the female stage and senescence. The amount of time between the female stage and senescence was much longer for the emasculated treatment than the pollinated treatments. This indicated that pollination triggered senescence. The full and partial pollination treatments were not significantly different in subset 1 (Table 6). The similar means between the full pollination and half pollination treatments shows that it does not take all of the stigma lobes to be pollinated in order for senescence to be triggered (Figure 4). Reduced longevity of flowers after pollination is beneficial to a plant from an evolutionary standpoint as it minimizes both resource expense and water loss (Weber & Goodwillie, 2007). Senescence of flowers lowers the energetic cost for plants. Once the flower produces offspring, termination is triggered. This indicates that self-fertilization will only occur in the absence of pollinator visitation. The half pollination treatment was applied on the very first day of the female stage. If self-fertilization occurred early on in the flowers life, then the control treatment would have shown a similar lifespan to the half pollinated treatment. Instead, the control showed a longer lifespan, indicating that autonomous selfing is delayed. This finding is further evidence of delayed selfing.

Together, these three experiments have provided a great deal of insight on the reproductive life of *Triodanis perfoliata.* Although self-fertilization of chasmogamous flowers alone cannot produce a full seed set, it is a reliable option for flowers to have. The experiments conducted support the idea of this species' usage of delayed selfing to reproduce. Flower pollination does trigger flower senescence, so self-fertilization may not even be used in some cases. However, in times that cross-fertilization cannot occur, self-fertilization is a delayed, but necessary option.

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