

DESCRIBING THE MATING SYSTEM OF *HALENIA DEFLEXA*, A NATIVE NEW
BRUNSWICK PLANT

By

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Abstract

The New Brunswick native plant *Halenia deflexa* (Gentianaceae) provides an excellent opportunity to investigate the association between within-plant variation in flower shape and within-plant variation in mating system. At the start of the season, *H. deflexa* flowers produced have long nectar spurs, but by the end of the season, nectar spur length declines significantly. This within-plant variation between early flowers and late produced flowers offers a character upon which natural selection could act. Nectar spurs offer a sugary reward to pollinators that then bring in outcross pollen for fertilization. Therefore, a decline in nectar spur length throughout the season could affect *H. deflexa* mating system. *Halenia deflexa* could be entirely self-incompatible, relying on outcrossing as its only mechanism for reproduction. On the other hand, *H. deflexa* could exclusively self or have a mixed mating system where both outcrossing and selfing are used. I compared plants with full pollinator access to pollinator excluded plants in bags to investigate this potential interaction between nectar spur and mating system. However, I observed no effect of pollinator access on *H. deflexa* fruit set or number of seeds per fruit throughout the season. I also compared emasculated flowers with anthers removed to intact flowers to observe the effects of autogamy on *H. deflexa* mating system. The ability to reproduce through autogamy increases fruit set throughout the season. Autogamous fruit produce more seeds than non-autogamous fruit early in the season, but by the end of the season there is no difference between the two. Collectively, my studies indicate that *H. deflexa* has a mixed mating system that does not rely on pollinators to successfully reproduce.

Introduction

Flowering plants exhibit an astonishing diversity of mating strategies. The complex and diverse mating strategies of plants arise to face the challenges of being sessile organisms that require a vector to facilitate transfer of pollen from male anthers to the stigma, the female receptive surface (Barrett and Harder 1996). Most flowering plants are hermaphroditic, producing both male and female organs, and therefore have the potential to self-fertilize, outcross or use a combination of both strategies in a mixed mating system (Barrett and Harder 1996, 2017; Kalisz and Vogler 2003; Goodwillie et al. 2005). Selfing occurs when male and female gametes from a single genetic individual fuse to form a zygote (Jarne & Charlesworth,

1993). It has been estimated that between 35-84% of plants are at least partially self-compatible, which means that self-fertilization is possible at least in principle (Jarne and Charlesworth 1993). However, self-compatibility does not necessarily mean that a plant will self-fertilize in nature, because plants may need a vector to transport self-pollen onto the female stigma. In contrast to selfing, outcrossing is the fusion of male and female gametes from two separate genetic individuals to form a zygote. Outcrossing in plants is mediated either by abiotic features such as wind or water, or biotic interactions where animals transport pollen for a floral reward (Friedman and Barrett 2009; Barrett and Harder 2017). A mixed mating system in plants uses both selfing and outcrossing to assure reproductive success (Goodwillie et al. 2005).

Mating systems have a direct impact on population genetics, gene flow and heterozygosity (Barrett 2003). Selfing, although relatively common among plants, can have drastic genetic effects such as high levels of homozygosity, which in turn cause inbreeding depression (Barrett and Harder 2017). Inbreeding depression can lead to reduced fitness, growth rate, and survival of offspring due to a lack of genetic diversity (Charlesworth and Willis 2009). Selfing rates are higher in annual species when compared to perennial species (Barrett 2003; Barrett and Harder 2017). An annual plant fulfils its entire lifecycle from seed to fruit in one single year, while perennial plants can produce flowers over several years. Outcrossing can also impact population genetics as it provides increased heterozygosity and increased genetic diversity (Barrett and Harder 2017). Nonetheless, outcrossing faces the challenge of pollen delivery to the female receptive surface often relying on wind or pollinators for transportation (Barrett and Harder 2017). Evolutionarily mixed mating strategies can provide the best of both worlds of outcrossing and selfing systems. Current estimates suggest that 42% of seed-producing plants employ a mixed mating strategy (Goodwillie et al. 2005).

Different mating system strategies employed by plants carry different costs and benefits. The benefits of outcrossing – increased heterozygosity, defense against mutations, and avoidance of inbreeding depression – are balanced against the costs of investing in flowers to attract pollinators and producing rewards for those pollinators (Willmer 2011). Moreover, relying on insects for pollination can be risky when they are unreliable. Even when outcrossing is achieved through wind pollination, the plant still incurs costs of excess pollen production because wind pollination is usually less precise and reliable (Friedman & Barrett, 2009). Plants can use autogamous selfing to assure reproduction even in unfavorable conditions (Goodwillie et al.

2005; Barrett and Harder 2017). Relying primarily or exclusively on self-pollen can alleviate the plant of any extra costs such as nectar production, floral pigment, fragrance, large floral displays, and size to attract pollinators (Proctor 1996; Goodwillie et al. 2010). However, selfing can be otherwise unfavorable due to allowing the genome to consist of two samples from a single parent, leading to inbreeding depression, increased homozygosity, and a reduction in the number of available ovules for outcrossing (Lloyd 1992a; Barrett and Harder 2017). Some plants exhibit physiological or morphological mechanisms to prevent selfing from producing viable offspring, these mechanisms can be costly but evidently producing self-offspring may be even more costly (Takayama and Isogai 2005).

Plants that rely on interspecific associations with animals as a primary mechanism of pollen transfer are 46.4% likely to exhibit a mixed mating system while abiotically pollinated plants only 26.9% (Goodwillie et al. 2005). When conditions are favorable and pollinators actively transport pollen, these systems can rely on outcrossing to reduce inbreeding depression (Vaughton and Ramsey 2010). However, as a back-up plan plants with a mixed mating system can autogaomously self when pollinators are unavailable (Lloyd 1992b; Kalisz and Vogler 2003; Vaughton and Ramsey 2010; Barrett and Harder 2017). In this way plants can have “the best of both worlds” of selfing and outcrossing, relying only on selfing secondary to outcrossing (Vaughton and Ramsey 2010). However, mixed mating systems are not effective in mitigating the costs of attracting pollinators to bring in self pollen among genetically identical flowers on the same individual – otherwise known as geitonogamy (Barrett and Harder 2017). Geitonogamy is a wasteful form of reproduction where pollen and ovules are lost to selfing when they would otherwise be outcrossed (Barrett and Harder 2017).

Mating strategies can vary within and between individuals and populations within a species, and they can also vary with changes in the abiotic environment (Barrett and Harder 2017). Mating system changes are often associated with changes in floral morphology. Selfing plants tend to have smaller flowers when compared to outcrossing flowers (Jarne and Charlesworth 1993; Goodwillie et al. 2010; Willmer 2011; Rifkin et al. 2019). Further, primarily selfing species tend to reduce the costs of pollinator attraction by reducing the number of flowers, pollen, anthers, ovules, and pigment (Willmer 2011). Morphological reductions from outcrossing species to selfing species are fairly common and are referred to as the selfing syndrome (Rifkin et al. 2019). In genus *Ipomoea*, for example, the primarily outcrossing species

I. cordatotriloba produces large flowers and copious nectar and pollen, while predominately selfing *I. lacunose* produces small flowers with less pollen and nectar (Rifkin et al. 2019). These same trends can be seen in sister species of *Capsella* (Sicard et al. 2011), *Mimulus* (Ritland and Ritland 1989), and *Arenia* (Wyatt 1984). There are clear trends between sister species that primarily outcrossing plants are morphologically different from primarily selfing plants.

Flower morphology can also vary within individual plants and this can affect their mating systems. Flowers produced later in the reproductive season are often faced with resource limitation and are allocated less energy and resources than flowers produced earlier in the reproductive season by the same plant (Ashman and Baker 1992; Kliber and Eckert 2004). As a result, late flowers on a plant may produce less pollen, fewer ovules, and smaller flowers (Ashman and Baker 1992). Furthermore, late flowers are more likely to use selfing than early flowers (Kliber and Eckert 2004). As late flowers are often smaller their anthers and stigmas may be closer in proximity than in larger early flowers (Kliber and Eckert 2004). Some plants produce flowers that never open to receive outcross pollen and rely entirely on self-pollination for fertilization (Lloyd and Schoen 1992). This extreme form of selfing in individual flowers is referred to as cleistogamy and often occurs alongside open “regular” chasmogamous flowers in individual plants (Lloyd and Schoen 1992). Cleistogamous flowers reduce the costs of attracting pollinators by simply remaining closed and are typically smaller and therefore morphologically different than chasmogamous flowers (Lloyd and Schoen 1992). With this information, it is clear that flower morphology can vary not only among species, but also within individual plants, and these morphological shifts can have impacts on reproductive success and mating systems.

The New Brunswick native plant *Halenia deflexa* (Gentianaceae) provides an excellent opportunity to investigate the association between within-plant variation in flower shape and within-plant variation in mating system (Figure 1). As an annual plant, *H. deflexa* has only one season to fulfill its lifecycle from germination to seed production. The mating system of *H. deflexa* has not been previously described, and due to this gap of knowledge, *H. deflexa* could have any variation of mating system from selfing, to outcrossing or a mixed mating system. On the one hand, small flower size and close proximity of anthers and stigma could indicate that selfing is occurring (Gillett 1963). On the other hand, *H. deflexa* flowers bear four relatively large nectar spurs which act as a reward for pollinators and can indicate allocation of energy to increase outcrossing (Gillett 1963; Hodges and Arnold 1995). Nectar spurs are an extension of

the corolla that house sugary nectar and are often associated with specialized pollinators such as hummingbirds, as well as an increased reproductive success (Hodges and Arnold 1995). The nectar reward is deeper within the corolla and forces close contact between pollinators and reproductive organs of the flower, and therefore has an effect on mating systems (Herrera and Pellmyr 2009). The nectar spurs on *H. deflexa* are reported to decrease in size throughout the season, with early flowers having the longest spur length and late flowers producing shorter, smaller spurs (Gillett 1963). This decrease could indicate that *H. deflexa* uses a mixed mating system relying on outcrossing in early season long spurred flowers and self-pollination in late season short spurred flowers. Given the reported change in nectar spur length, I hypothesize that *Halenia deflexa* reliance on self-pollination will increase over the flowering season.

My honours research aims to characterize the mating systems of *H. deflexa*, and test whether the mating system changes along with flower shape within plants throughout the season. Specifically, my goals were to : (1) Quantify within-plant changes in floral spur length and flower size using digitized herbaria records ; (2) Determine whether *H. deflexa* is capable of self-fertilization using hand pollinations in the field ; (3) Test whether frequency of pollinator-mediated fertilization decrease over season through field manipulations that (a) excluded or included pollinator access to plants throughout the flowering season, and (b) prevented or allowed autogamous reproduction.

As *H. deflexa* has never been scientifically explored, my honours will act as the base of knowledge on its mating systems and will allow further research to be conducted on this unique plant. Reproduction in plants affects the movement of genes within a population, especially in annual plants (Barrett 2003; Willmer 2011). These genetic movements then affect the evolutionary dynamic and divergence in speciation (Barrett 2003). Studying the interactions between mating system and changes in flower morphology throughout the season allows for further investigation into the causes and consequences of within-plant variation in floral shape in *Halenia deflexa*.



Figure 1: Photograph of *H. deflexa* demonstrating nectar spurs. Image taken by Sarah Neima.

Methods

Study System

Halenia deflexa (Sm.) Griseb. commonly, Spurred Gentian, is a native species to New Brunswick. *Halenia deflexa* is a late flowering annual plant that grows on rocky river shores, in cool damp woods and glades (Gillett 1963). Its flowers produce four nectar spurs that, according to field guides and other gray literature, are longer on the flowers at the start of the flowering season but appear to be short or non-existent in late season flowers (Gillett 1963). These spurs are reported to range from 0.3-0.5cm in length (Gillett 1963). Several of the late season flowers appear to be cleistogamous, meaning these flowers remain closed never opening at all and rely on self-fertilization. *Halenia deflexa* arranges its flowers into a cyme inflorescence with uppermost flowers opening first and flowering then progressing down the plant. The fruit are capsules that disperse by opening at the apex. The flowers are relatively small from 0.8 - 1.4 cm in length (Gillett 1963). This plant is relatively understudied in scientific literature.

Quantifying within-plant change in flower shape (Goal 1)

Digitized herbarium sheets of *Halenia deflexa* from NYBG, UOG, ACAD, UNB and UOC were used to observe changes in morphology over time, from early- to late- flowers within plants. Digitized herbarium sheets allow a larger sample size to be studied across a large area and period of time; they are becoming more commonly used in scientific research to track the effects of climate change on plants (Davis et al. 2015; Meineke et al. 2018). In order to analyze the changes in size of flowers I used ImageJ (Schneider et al. 2012). Two flowers were analyzed in each herbarium specimen, one upper and one lower both from the same plant. *Halenia deflexa* like many other plants, flowers from top to bottom, therefore comparing an upper and lower flower allows examination of an early season flower (upper) and a later season flower (lower). The spur length and flower length of each upper and lower flower was recorded as well as the length of the first internode and the internode below first flowering inflorescence. All together two reproductive characters (upper and lower flower) and a vegetative character (internode length) were recorded from each herbarium sheet. Location, date and habitat were also recorded from the label on each herbarium sheet. This was a project done in collaboration with a previous student (Megan Sanderson) who examined 63 herbarium sheets; I added an additional 63 to the dataset.

All recorded information from each herbaria sheet was analysed using a mixed model comparing the within and between plant variances of spur length, flower length and internode length (Bates et al. 2015). This allows an estimate of how much variation is occurring within plants and between plants for those particular traits. Within this analysis I expected there to be a decline in spur length, flower length and internode length within plants. In previous reports spur length was noted to decline, so I would expect the largest amount of within plant variation to occur within this trait. Analysis of spur length was performed by Emily Austen, and I then repeated this analysis for both flower length and internode lengths. Plant ID is a random predictor in this analysis as two measurements were taken from each herbaria specimen. Position is the fixed predictor in the analysis that takes into account positional effect, this allows for early produced organs and late produced organs to be compared.

Field Experiments (Goals 2 and 3)

All experiments were conducted on one population of *H. deflexa* from the Nepisiguit River over one reproductive season from the end of June until the start of September 2019. This population was found using GIS maps from the Atlantic Canada Conservation Data Center and was spread across the south shore riverside of the Mi'gmaq trail from access point K and access point L found on the Mi'gmaq Trail association website under the maps section. Each experimental plant was given a unique code indicating its location (Site K or L), number and treatment (Open or Bag) on a sharkskin tag. Leaf tissue was taken from each of the experimental plants and was stored in silica gel envelopes for future genetic testing to determine parentage of seeds produced during the experiment. A summary table of all the manipulations for the field experiments is available (Table 1).

Table 1: Summary table of all manipulations conducted on *H. deflexa* in field manipulation. Possible reproductive strategies: Geitonogamy = G, Autogamy = A, Outcrossing = O/C.

Plant Treatment	Flower Manipulation	Pollinator Access	Possible Reproduction	Experiment
Bag	Self	No	A	Goal 2
Bag	Outcross	No	O/C	Goal 2
Bag	Handle	No	A	Goal 3b
Open	Emasculate	Yes	O/C + G	Goal 3a
Open	Handle	Yes	O/C + G + A	Goal 3a and b

Testing for self-compatibility (Goal 2)

To test if *H. deflexa* is self-compatible I hand pollinated several flowers in pollinator exclusion bags. A total of 38 plants from the Nepisiguit river were placed in 45cm (h) by 25cm (w) pollinator exclusion bags sewn from mesh allowing air and water exchange but preventing pollinators from visiting the plant. These bags were placed on dowels and tied around the bottom using string to restrict access to the plants from the ground. Each plant was labeled using a unique code. In order to observe a large amount of variation between early and late season flowers, large plants were selectively chosen and bagged to be used in both goal 2 and 3.

Within each bagged plant two manipulations were completed on separate flowers: self-pollination (N=37) and outcross pollination (N=35). In each treatment, manipulations were done on bud stage flowers before anthers had begun dehiscing. The outcrossing and self-manipulations were performed on the same date whenever possible (N=7), in cases where they could not be completed on the same day they were completed as close to first treatment date as possible (mean(SD)= 7.75 days (3.25)), and in all plants they were completed in a random order.

The selfed and outcrossed manipulations both began with an emasculation, which is the removal of the pollen producing anthers before they dehisce to prevent selfing. Due to the small size of the anthers a magnifying visor was worn, and forceps were used to remove the four anthers. Each manipulated flower had a unique coloured embroidery thread tied around its pedicel. No thread colour was used twice on the same manipulated plant. From there the selfed flowers were manually selfed using forceps and a dehiscing anther from another flower on the same plant. As often as possible selfing was conducted on the same day that emasculations were completed (mean(SD) date between manipulations =0.276(0.538)). Outcrossed flower manipulations are manually outcrossed with a dehiscing anther from a *H. deflexa* individual at a distance of at least five meters away from sample plant. Similar to selfing plants outcrossing was completed on the same day as emasculations (mean(SD) date between manipulations =1.29(0.591)). If pollen is compatible and plant has sufficient resources the hand pollinated flower should develop into a fruit.

I returned to collect the fruits into labeled envelopes before they opened to release their seeds. Each fruit was measured in length, any signs of herbivory were recorded, and the total number of seeds were counted. The self- and outcross-manipulations allow self-compatibility to be tested and estimate expected fruit and seed production per flower from a single pollen donor. If *H. deflexa* is fully self-compatible then there would be no significant difference in fruit set (proportion of fruit produced to flowers produced) or number of seed per fruit of outcrossed or selfed manipulations.

In order to analyse these results, I used a generalized linear mixed model in lme4 to compare the fruit set (proportion of flowers that produced fruit) of outcross and self-hand pollinations (Bates et al. 2015). For the number of seeds produced per fruit, I used a similar model to logistic regression using the quassipoisson family (R Core Team 2019). Originally, I had intended on using a poisson generalized linear mixed model but the model was over

dispersed and the quasipoisson fixed that issue. In both analyses, flower manipulation (self or outcross) is a fixed predictor. In order to get p-values for the lme4 regression, I used the lmerTest package (Kuznetsova et al. 2017).

To further analyze *H. deflexa* self-compatibility, the bagged plants (N=38) were paired with an open un-bagged plant (N=40, 2 plants unpaired) to observe how many unmanipulated, unmarked flowers would produce fruit in each treatment. Fruit produced in bagged plants is a result of autogamy without pollinators, while fruit produced on open plants are a result of selfing, geitonogamy or outcrossing facilitated by pollinators. A higher fruit set in open plants would suggest that *H. deflexa* is at least partially self-incompatible, while a similar fruit set in both conditions would indicate that *H. deflexa* is self-compatible.

The 78 plants were collected at the end of the season and were placed in labeled paper bags and stored in lab. In lab they were examined for proportion of total fruit set to total unmanipulated flowers produced as well height, number of nodes and flowering nodes. In order to test if access to pollinators has an effect and if *H. deflexa* is fully self-compatible, a logistic regression was used to compare fruit set of open versus bagged plants (R Core Team 2019). The fruit set is the proportion of flowers that produced fruit, and the treatment of open or bag is the fixed predictor. Although seeds were not counted per fruit in this experiment they were collected and placed in labeled envelopes for future germination and genetic analysis.

Evaluating reliance on autogamy from first-to-last flowers (Goal 3)

Each bagged plant from the experiment addressing Goal 2 was paired with an open (i.e., un-bagged) plant of similar size and in close proximity. These plants were actively selected for large size and height in order to ensure flowers would be available for manipulations for a long period of the flowering season. Using larger plants allows for the examination of several flowers from the same individual rather than using small plants that are more likely to produce fewer flowers. A total of 38 bagged plants and 40 open plants (2 open plants were unpaired) were used in evaluating *H. deflexa* reliance on autogamy throughout the season. While the bagged plants exclude pollinators, the open plants remain accessible to pollinators. In order to evaluate reliance on autogamy from first to last flowers I used two complementary analyses. The first analysis in goal 3 uses only open plants to examine temporal trends in fruit set and seed production of emasculated versus intact (handled) flowers. The second analysis examines fruit set and number

of seeds per fruit in marked-but-unmanipulated flowers on open-pollinated *versus* bagged plants to ask if the effect of pollinator access changes throughout the season. Marking the unmanipulated flowers allows me to roughly estimate which day the flower opened. Within the open and bagged treatments several manipulations were made to individual flowers of these sample plants. In each treatment, manipulations were done on bud stage flowers before anthers had begun dehiscing, as in Goal 2 I used coloured embroidery thread to identify manipulations.

For the first analysis, two manipulations were repeated on several flowers within each open plant over the course of the flowering season. These manipulations were emasculate and handle. The emasculation treatment entails the removal of the flower's anthers using forceps, while the handle control manipulates the flower by opening the petals and touching but not removing the anthers. An emasculated flower on an open plant can only set fruit if a pollinator brings pollen to the stigma. Pollinators can transport pollen from another plant in the population (outcross) or from another flower on the same plant (geitonogamy). A handled treatment can set fruit using its own pollen (selfing), or pollen transported by pollinator (outcross or geitonogamy). All manipulated flowers are collected at the fruit stage and placed in labeled envelopes. In lab, each fruit was measured in total length, any signs of herbivory were recorded, and the total number of seeds were counted. The emasculation treatment will allow for the combined rates of geitonogamy and outcrossing to be estimated, while the handle treatment will allow for the combined rates of autogamy, geitonogamy and outcrossing. The handle treatment further acts as a control, and allows us to observe if handling of the flowers damages them. Comparing fruit set and seed number of the emasculated (Geitonogamy + Outcross) versus handled control (Geitonogamy + Outcross + Autogamy) allows rates of autogamy to be estimated. Because flower manipulations were repeated as frequently as possible, and in most cases several times on the same plant, I was able to observe how the fruit set and the number of seeds produced changes with time of season for both manipulations. If reliance on autogamy increased over time, then I would expect to see a higher probability of fruit set and seed number in the handle control when compared to the emasculated manipulation over time. I used a generalized mixed effects logistic regression to test this prediction on fruit set (Bates et al. 2015). As for number of seeds produced, I attempted to fit the count data to a poisson model, however the model was over dispersed. In order to get a better fit, I used a generalized linear mixed effects model under the normal distribution (Bates et al. 2015). In order to get p-values for the lme4 regression, I used the

lmerTest package (Kuznetsova et al. 2017). In these models, site, flower manipulation and centered treatment date are fixed predictors. Site is the location of the plant in either site K or site L both of which are along the Nepisiguit river. Flower manipulation is either emasculated or handled. Centered treatment date is the date that a flower was manipulated, where day 0 is the first date of manipulations on the population. I also included an interaction between treatment date and flower manipulation to examine if the effect of flower manipulation depends on treatment date. As most plants in this experiment had several manipulated flowers, I included plant ID as a random factor to take into account some of this variation. If any of these predictors were non-significant ($P > 0.05$) they were removed from the model.

To examine the effect of pollinators on fruit set and number of seeds per fruit throughout the season, I marked several flowers on bagged pollinator-excluded plants and open plants with pollinator access. The open handle flowers were the same flowers used as handle controls for the comparison of emasculated versus handled flowers (see above), and the bagged flowers were handle manipulated in the same way as described above for the open handles. The bag handle manipulation allows for the examination of rates of autogamy without pollinator facilitation as there is no geitonogamy or outcrossing that can occur within the bagged treatments. While the open handle treatments allowed examination of the combined rates of autogamy, geitonogamy and outcrossing as these flowers have access to pollinators. An occasional flower (N=6) was emasculated without follow-up pollination within the bag treatment. These flowers can be used to ensure that no pollinators were getting into the exclusion bags because they will have no other possible method to receive pollen. Comparing the bag handles flower (autogamy only) versus open handle flowers (autogamy, geitonogamy, and outcrossing) allows an estimation of the combined rate of geitonogamy and outcrossing without autogamy.

As in the previous goal, the fruit set was analyzed using a generalized mixed effects logistic regression (Bates et al. 2015). If pollinators are having an effect on fruit set of *H. deflexa* I would expect to see a higher fruit set in the open handle flowers. The number of seeds per fruit was originally analyzed using a generalized poisson mixed effects model, however that model was over dispersed. Instead I ended up using a generalized linear mixed effects model under the normal distribution (Bates et al. 2015). In order to get p-values for the lme4 regression, I used the lmerTest package (Kuznetsova et al. 2017). These estimates are weak because they do not differentiate between geitonogamy and outcrossing. In the future the seeds will be used in

genetic analysis to compare offspring genotype to maternal plant genotype in order to differentiate between outcrossing and geitonogamy. In these pollinator models, flower treatment and centered treatment date are fixed predictors. Site was not considered in the fruit set model as 100% of flowers set fruit in the open treatment at site K resulting in an issue with singularity. Therefore, I removed Site to eliminate this issue, however I was able to keep Site as a fixed predictor for the seed analysis. Flower manipulation is either open handle with pollinator access or bag handle without pollinator access. Centered treatment date is the date that a flower was manipulated, where day 0 is the first date of manipulations on the population. I also included an interaction between treatment date and flower manipulation to examine if the effect of flower manipulation depends on treatment date. As most plants in this experiment had several manipulated flowers, I included plant ID as a random factor to take into account some of this variation. If any of these predictors were non-significant ($P > 0.05$) they were removed from the model.

Results

Quantifying within-plant change in flower shape (Goal 1)

The purpose of this experiment was to quantify the within-plant changes in flower length, spur length and internode length in *H. deflexa* herbaria specimens (N=126). I first examined if each of these organ-level traits exhibit more variation within plants or among plants. Spur length varied more within plants than among plants (Table 2a). The interclass correlation coefficient of just 9.6% reveals that >90% of the total variation in spur length is attributable to the differences among flowers within plants. The within-plant variation in spur length was partly explained by a tendency for spur length to decrease from upper flowers to lower flowers in the same plant (Figure 2A). The spurs of upper flowers (i.e., the first flowers to open on the plant) are on average 0.67 mm longer than those of lower flowers (which open later in the season). After accounting for this positional effect, 76% of the remaining variation in spur length was attributable to variation among flowers within plants, while 24% of the remaining variation was attributable to variation in mean spur length between plants (Table 3a).

Similarly, the total variation in flower length (excluding spur) was mostly attributable to variation among flowers within plants, rather than variation within plants (Table 2b). The

interclass correlation shows that only 0.89% of the variation in flower length is due to variation among plants before taking into account position of flower along the plant. Upper early season flowers are, however, roughly one-third longer than lower late season flowers (Figure 2b), and after accounting for this positional effect, the remaining variation in flower length is 45.2% attributed among plants and 54.8% within (Table 3b). Within plant variation in flower length is therefore better explained by position than is within plant variation in spur length.

Variation in internode length, much like spur and flower length, is greater within plants than among plants before taking into account positional effects (Table 2c). The interclass correlation shows that <1% of variation before taking into account position is attributed to among plant variation. Lower, early-season internodes are on average 2.54 mm longer than upper late season internodes (Figure 2c, Table 3c). After taking into account positional effect, only 36.2% of variation remained attributable to within plant effect in internode length (Table 3c). The results differ from spur length where a much larger amount of variation remained attributable to within plant variation.

Table 2: Hierarchical model coefficients and test statistics for spur length, flower length and internode length on *Halenia deflexa*. All coefficients are reported on scale of model fit. LCI indicated lower 95% confidence interval, UCI indicates upper 95% confidence interval. An asterisk indicates a significant difference (P <0.05).

	a. Spur Length	b. Flower Length	c. Internode Length
<i>Random effects</i>	Variance	Variance	Variance
Plant ID	0.078	0.029	0.038
Residual	0.732	3.261	6.003
<i>Fixed Effects</i>	Coef (LCI, UCI)	Coef (LCI, UCI)	Coef (LCI, UCI)
Intercept	2.72 (2.59, 2.84)*	6.98 (6.73, 7.22)*	4.00 (3.69, 4.31)*
<i>N</i>	222 Spurs on 126 plants	222 Flowers on 126 plants	250 Internodes on 126 plants

Table 3: Hierarchical model coefficients and test statistics for spur length, flower length and internode length on *Halenia deflexa* taking into account positional effect. All coefficients are reported on scale of model fit. LCI indicated lower 95% confidence interval, UCI indicates upper 95% confidence interval. An asterisk indicates a significant difference ($P < 0.05$).

	a. Spur Length	b. Flower Length	c. Internode Length
<i>Random effects</i>	Variance	Variance	Variance
Plant ID	0.165	1.062	1.608
Residual	0.533	1.287	2.830
<i>Fixed Effects</i>	Coef (LCI, UCI)	Coef (LCI,UCI)	Coef (LCI,UCI)
Intercept (early-produced organ)	3.01(2.86, 3.15)*	7.82 (7.56, 8.09)*	2.74 (2.37, 3.11)*
Late-produced organ	-0.67(-0.86, -0.47)*	-2.01 (-2.32, -1.69)*	2.54 (2.11, 2.96)*
<i>N</i>	222 Spurs On 126 plants	222 Flowers on 126 plants	250 Internodes on 126 plants

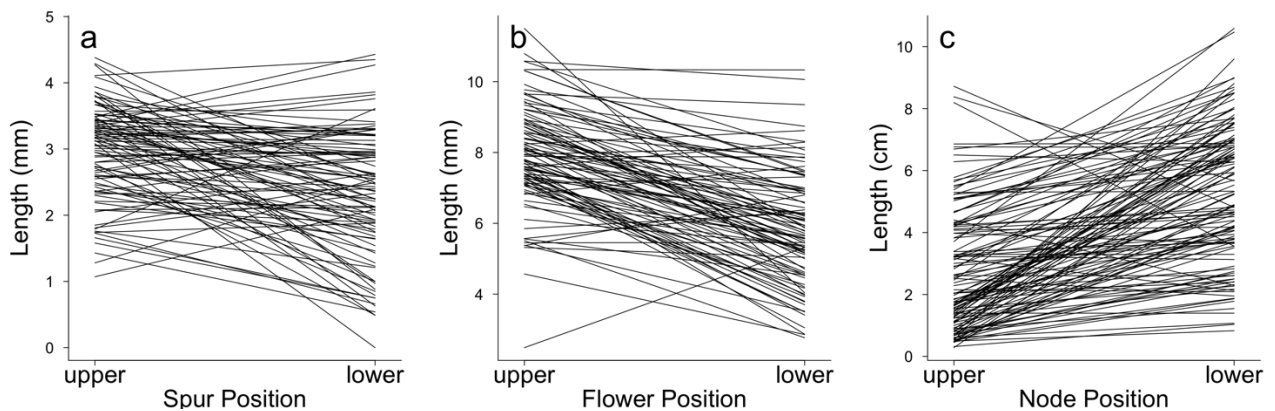


Figure 2: Variation in spur length (a), flower length (b) and internode length (c) in upper and lower positions along *Halenia deflexa*. Each line on the figures represents a single plant (n=126).

Testing for self-compatibility (Goal 2)

I used hand-pollinations and pollinator exclusion bags to test for self-compatibility of *H. deflexa*. Throughout the flowering season a few flowers (N=6) were emasculated within the pollinator exclusion bags without subsequent hand pollination. These flowers never set fruit, confirming that no pollinators were able to pollinate those flowers and that the bags are effective.

Hand-pollinations were performed using self-pollen and using outcross pollen. A higher fruit set of outcross-pollinated flower than of self-pollinated would suggest that *H. deflexa* is not fully self-compatible. Roughly 80% of selfed flowers set fruit (mean (SD) = 0.811 (0.397), N=37), definitively proving that *H. deflexa* is self-compatible. Moreover, the fruit set of selfed flowers was unexpectedly significantly higher than that of outcrossed flowers (mean (SD) = 0.543 (0.505), N=35, Table 4a). Selfed flowers also produced more seeds per fruit (mean(SD) = 12.23(5.35)) than did outcrossed flowers (mean(SD)= 10.47(5.49)) however the difference is not statistically different (Table 4b).

Table 4: Generalized linear model coefficients and test statistics for hand pollinations of *Halenia deflexa* in pollinator exclusion bags. All coefficients are reported on scale of model fit. An asterisk indicates a significant difference (P <0.05).

<i>Fixed effects</i>	a. Fruit set Model Family: Binomial		b. Number of seeds Model Family: Quassipoisson	
	Coefficient (+/- SE)	z-value (p-value)	Coefficient (+/- SE)	t-value (p-value)
Intercept (Outcross Pollination)	0.172 (0.339)	0.506 (0.6125)	2.349 (0.1131)	20.771 (<0.01)*
Self-Pollination	1.283 (0.540)	2.378 (0.0174)*	0.155 (0.141)	1.106 (0.274)
<i>N</i>	35 Outcrossed 37 Selfed		35 Outcrossed 37 Selfed	

Capacity for self-pollination was also tested by comparing the fruit- and seed-set of unmanipulated flowers in pollinator exclusion bags to that of unmanipulated flowers with access to pollinators. A higher fruit set in plants with access to pollinators would indicate that *H. deflexa* relies, at least partially, on outcross pollination. Fruit set probability was higher in open plants (mean (SD) = 0.56 (0.16)) with pollinator access than in bagged plants (mean (SD)) = 0.51(0.18)), however that difference was not statistically significant (p=0.147, Table 5). This goes to show that plants can produce a similar amount of fruit autogamously without pollinators as plants that have access to pollinators. It is clear that *H. deflexa* does not have physical or physiological mechanism to prevent selfing and does not need outcross pollen to successfully reproduce.

Table 5: Logistic model coefficients and test statistics for unmanipulated *Halenia deflexa* flowers on pollinator excluded and pollinator access plants . All coefficients are reported on scale of model fit. Asterisk indicates significant difference (P<0.05).

<i>Fixed effects</i>	Coefficient (+/- SE)	z-value (p-value)
Intercept (Bag- pollinator excluded)	-0.658 (0.057)	-11.6 (<0.01)*
Open- pollinator access	0.108 (0.075)	1.45 (0.147)
<i>N</i>	38 open plants (915 flowers) 40 bag plants (1145 flowers)	

Evaluating reliance on autogamy from first-to-last flowers (Goal 3)

My final goal focuses on examining if *H. deflexa*'s mating system changes throughout the season. I predicted that *H. deflexa* reliance on pollinator mediated fertilization will decrease throughout the flowering season and therefore reliance on autogamy would increase. I addressed this question using two complementary analyses. The first analysis examines temporal trends in the fruit set and seed production of emasculated *versus* intact (handled) flowers on open-pollinated plants. The second examines fruit set and production of handled flowers on open-pollinated *versus* bagged plants to ask if the effect of pollinator access changes throughout the season.

The first analysis compared the fruit set and seed set of emasculated *versus* non-emasculated (handle) flowers on open-pollinated plants. An increased reliance on autogamy later on would be indicated by an interaction between flower manipulation and treatment date. The non-emasculated handle flowers that are able to reproduce through autogamy, outcrossing and geitonogamy had a higher fruit set (mean(SD) handle fruit set= 0.86(0.35), Table 6a) than the emasculated flowers that are only able to reproduce through outcrossing and geitonogamy (mean (SD) emasculate fruit set = 0.70(0.16), Table 6a, Figure 3). There was, however, no interaction between treatment date and flower manipulation, therefore there was no evidence that reliance on autogamy increased from first-to-last flowers on plant (Figure 3, Table 6a). In all manipulations there was a decline in the fruit set from start of the season to the end of the season (Table 6a, Figure 3). Site K produced more fruit than site L (Table 6a, Figure 3). Overall these results indicate that the ability to reproduce autogamously improves fruit set in *H. deflexa*.

Similar to the fruit set, the number of seeds per fruit declined throughout the season (Figure 4). However, there is an interaction between treatment date and flower manipulation

(Table 6b, Figure 4). The interaction coefficient is positive and indicates that the effect of date on seeds per fruit was weaker for the emasculated flowers that can only reproduce through outcrossing and geitonogamy than it was for the handled flowers (Table 6b). Therefore, early in the season, handled flowers produce many more seeds per flower than do emasculated flowers, but by the end of the season, there is no longer a difference between treatments (Figure 4). As for fruit set, these results indicate that the ability to reproduce autogamously improves a flower's reproductive success in *H. deflexa*, at least in the early part of the season.

Table 6: Logistic model coefficients and test statistics for open pollinator access *Halenia deflexa* plants. All coefficients are reported on scale of model fit. Significant differences are indicated by asterisk next to p-value ($P < 0.05$).

<i>Fixed effects</i>	a. Fruit set Family: Binomial		b. Number of seeds Family: Normal	
	Coefficient (+/- SE)	z-value (p-value)	Coefficient (+/- SE)	t-value (p-value)
Intercept (Handle)	4.268 (0.961)	4.443(<0.01)*	22.874 (1.554)	14.724 (<0.01)*
Treatment Date	-0.0614(0.029)	-2.088(0.037)*	-0.541(0.068)	-7.911 (<0.01)*
Emasculate	-1.309 (0.510)	-2.566 (0.010)*	-7.725 (2.003)	-3.856 (<0.01)
Site L	-1.520(0.587)	-2.590(<0.01)*	NA	NA
TreatmentDate *Emasculate	NA	NA	0.251(0.096)	2.610 (0.011)*
<i>Random effects</i>	Variance		Variance	
Plant ID	0.492		7.694	
Residual	NA		14.865	
<i>N</i>	72 Handle flowers on 39 Plants 71 Emasculate flowers on 36 Plants		62 Handle flowers on 35 Plants 50 Emasculate flowers on 28 Plants	

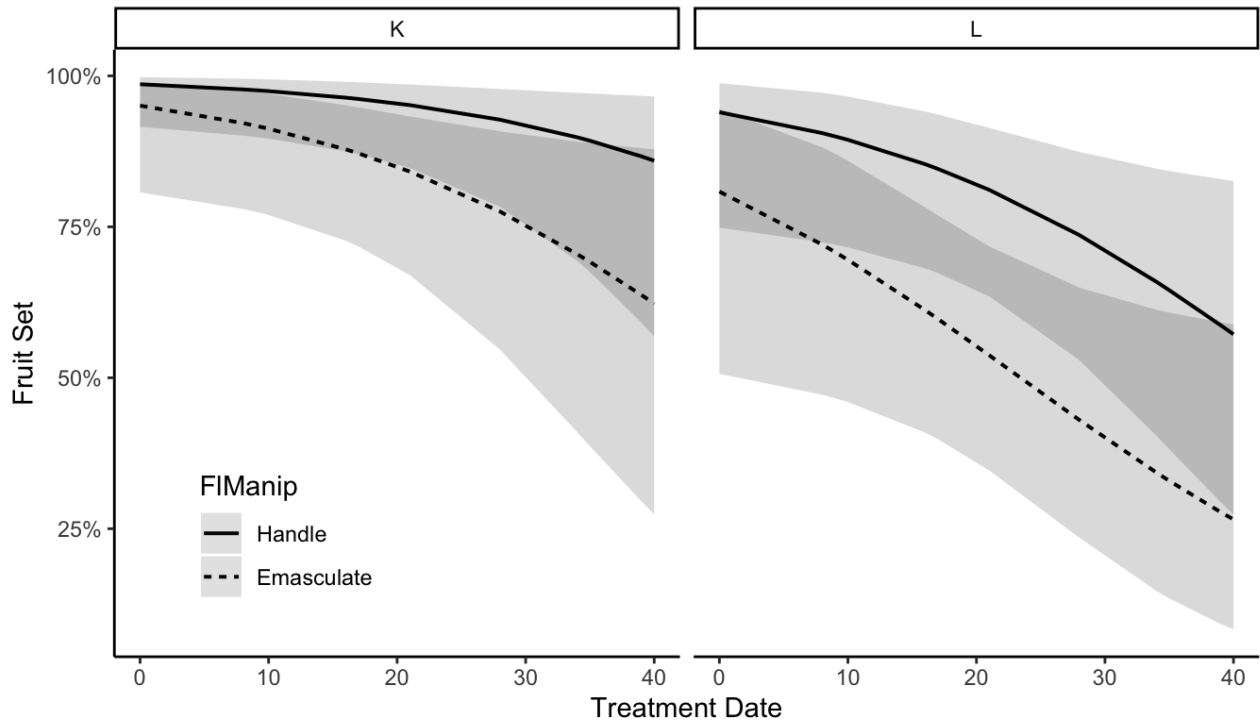


Figure 3: Predicted probability of fruit set on open plants in both site K and L, in handled and emasculated flowers throughout the flowering season. Shading indicates the 95% confidence interval.

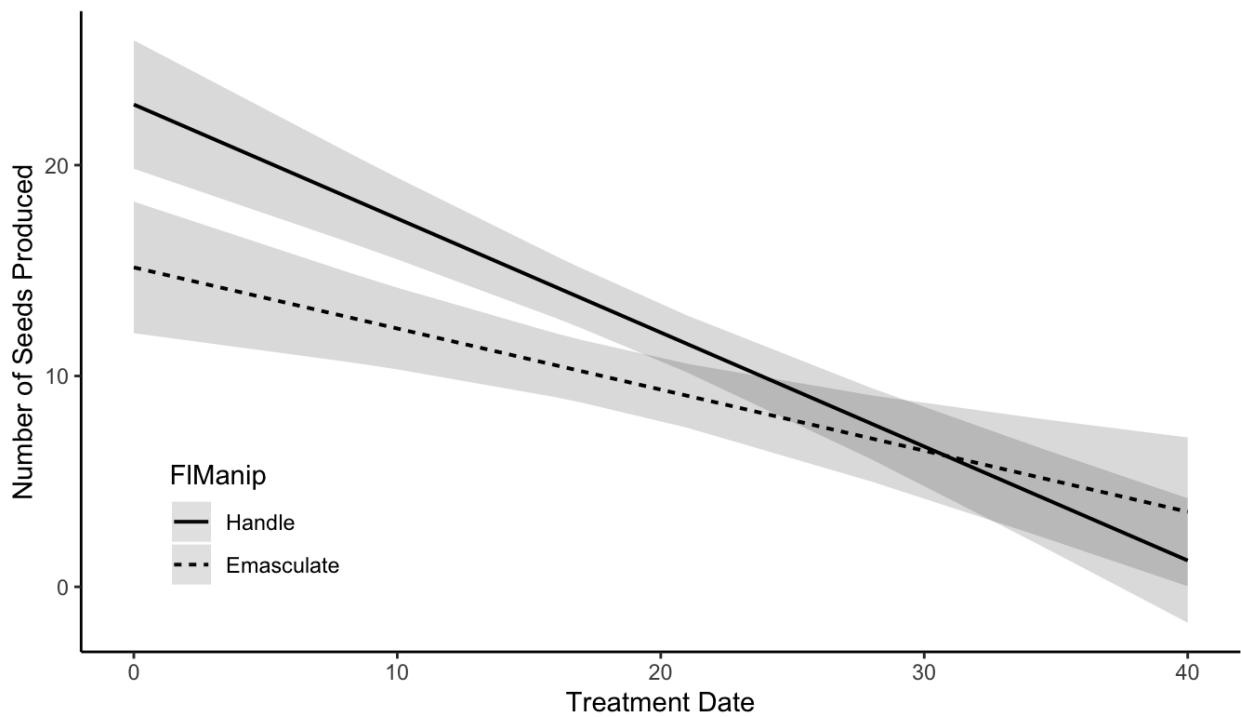


Figure 4: Predicted number of seeds per fruit on open plants in handled and emasculated flowers throughout the flowering season. Shading indicates the 95% confidence interval.

The second analysis evaluating temporal changes in a plant's reliance on autogamy made use of handled flowers on both bagged and open-pollinated plants. Because I know the approximate date of flower opening for each of these marked flowers, I can use them to test (1) whether flowers with access to pollinators generally set more fruit or seed than those without pollinators, and (2) whether the difference in fruit or seed set between these two treatments changed with time. I removed site as a predictor in this analysis because there were issues with singularity as one treatment group at a site produced fruit 100% of the time leaving no error to be examined. I found no overall effect of treatment (open *versus* bag) on a flower's probability of fruit set (mean(SD) fruit set open pollinator access = 0.86(0.35) , mean(SD) bag pollinator excluded = 0.80(0.40), Table 7a). There was, however, a significant negative effect of date on fruit set (Table 7a, Figure 5), but no interaction between date and treatment. This means that the probability of a flower maturing into a fruit decreased throughout the season but both treatments were affected similarly throughout the season.

Again, I had difficulty fitting the count data for number of seeds per fruit on a Poisson distribution, so I fit it using a normal distribution (Table 7b). Similarly, seed per fruit did not differ between treatments (mean(SD) pollinator access = 11.92(5.20), mean(SD) pollinator excluded = 12.41(5.25), Table 7b, Figure 6). Therefore, pollinator access did not affect the number of seeds produced and site did not affect seeds per fruit (Table 7b). Flowers manipulated later in the season produced fewer seeds than flowers manipulated earlier in the season in both treatments (Table 6, Figure 7b). All together these results signify that the relative importance of self-fertilization does not increase over the season, and that pollinators are not essential in reproductive success of *H. deflexa*.

Table 7: Logistic model coefficients and test statistics for handled *Halenia deflexa* plants taking into account multiple predictors. All coefficients are reported on scale of model fit. Significant differences are indicated by asterisk next to p-value ($P < 0.05$).

<i>Fixed effects</i>	a. Fruit set Family: Binomial		b. Number of seeds Family: Normal	
	Coefficient (+/- SE)	z-value (p-value)	Coefficient (+/- SE)	t-value (p-value)
Intercept (Bag Treatment)	4.311(1.091)	3.951(<0.01)*	21.711(1.215)	17.861(<0.01)*
Treatment Date	-0.114 (0.035)	-3.273(<0.01)*	-0.446 (0.046)	-9.621(<0.01)*
Open Treatment	0.402 (0.533)	0.756 (0.450)	-0.391(0.853)	-0.459 (0.648)
Site	NA	NA	-0.830 (0.880)	-0.943 (0.349)
TreatmentDate* OpenTrt	NA	NA	NA	NA
<i>Random effects</i>	Variance		Variance	
Plant ID	0.543		4.462	
Residual	NA		12.836	
<i>N</i>	76 flowers in 34 bag plants 72 flowers in 39 open plants		61 fruits from 31 bag plants 62 fruits from 35 open plants	

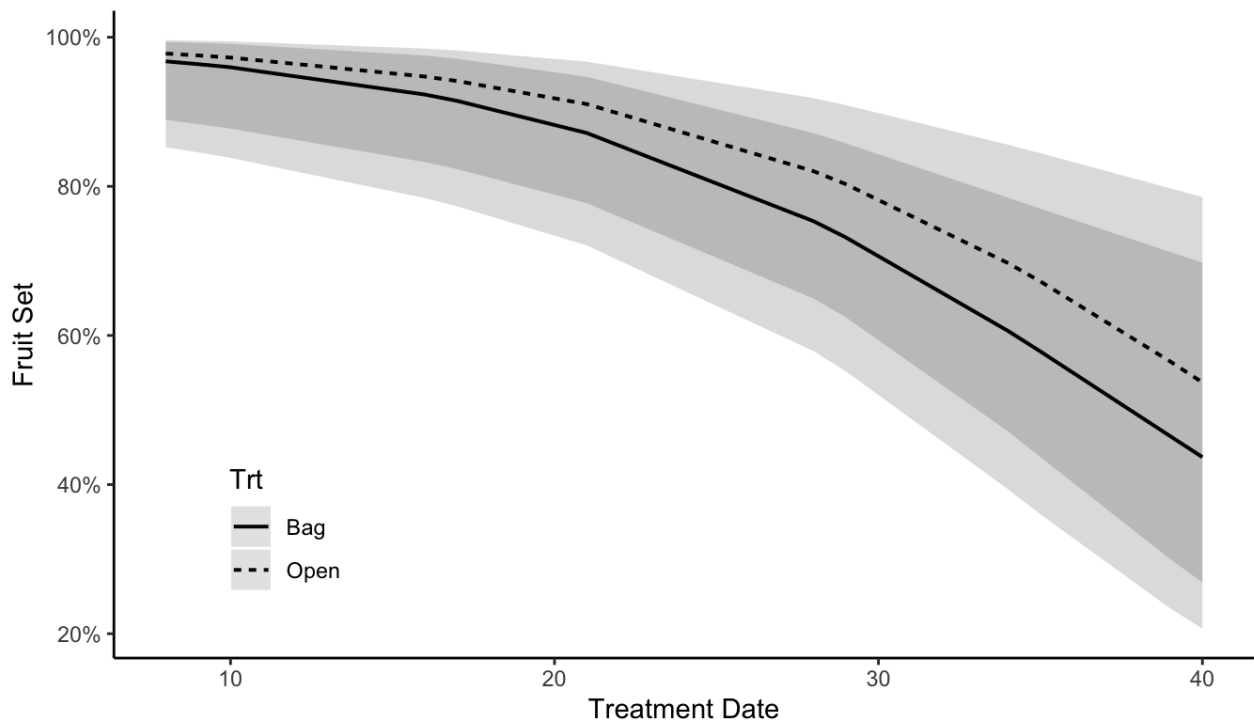


Figure 5: Predicted probability of fruit set on bagged and open *H. deflexa* flowers throughout the flowering season. Shading indicates the 95% confidence interval.

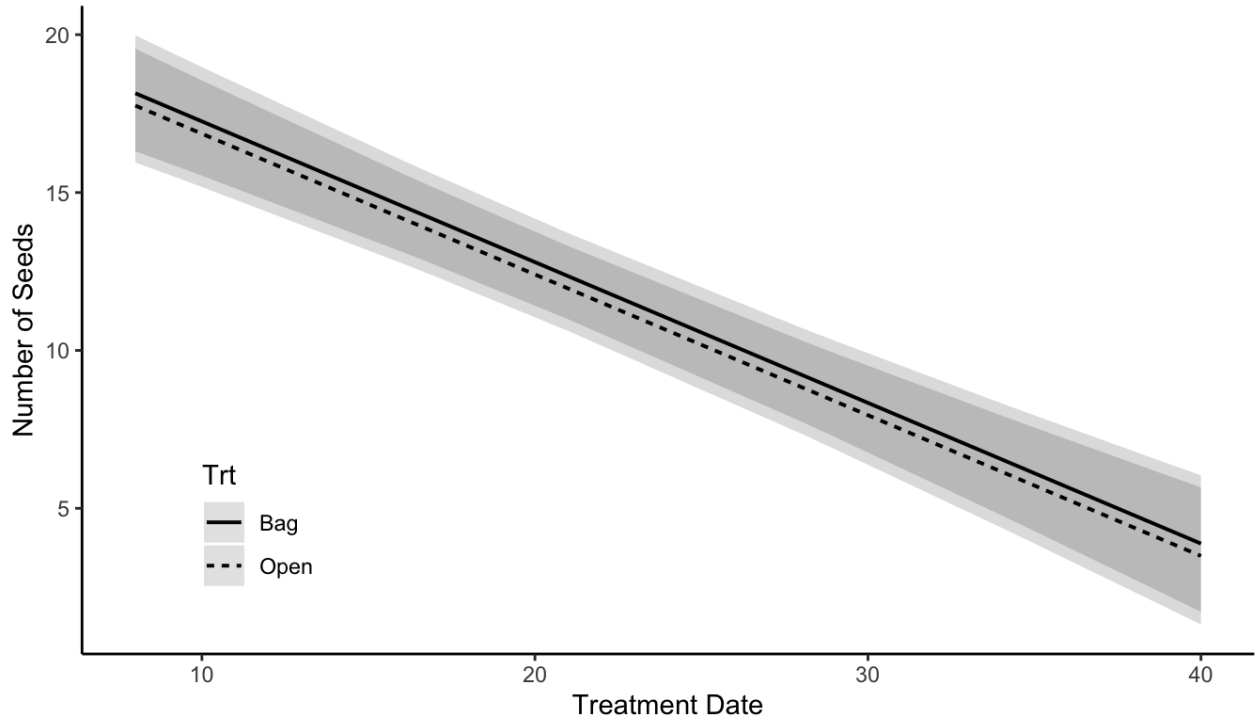


Figure 6: Predicted probability of seed per fruit on pollinator excluded bagged *H. deflexa* flowers and pollinator access open *H. deflexa* flower throughout the flowering season. Shading indicates the 95% confidence interval.

Discussion

As the mating system of *H. deflexa* has never been investigated my primary goal was to examine if the mating system shifts along with a decrease in nectar spur length. I first confirmed that nectar spur length does in fact decline through the season and determined that most variation in spur length comes within the plant rather than among individuals. I then determined that *H. deflexa* is able to successfully reproduce through autogamy and does not have physiological or physical mechanisms to avoid selfing. Finally, I examined how the mating system can change throughout the season. When able to reproduce through autogamy, *H. deflexa* produces more fruit throughout the season. Within those fruit, the number of seeds produced in flowers able to use autogamy declines more from beginning of the season to the end of the season than in flowers unable to use autogamy. This indicates that although autogamy may allow for more seeds to be produced early in the season, there is no difference between treatments by the end of the season. Pollinator access does not have an effect on fruit set or number of seeds produced.

Although *H. deflexa* does have indication that pollinators do visit and bring in pollen as several emasculated flowers produced fruit, pollinators are not essential in reproductive success of *H. deflexa*. Overall, I was able to explore the complex mating system of *H. deflexa*.

Quantifying within-plant change in flower shape (Goal 1)

I analyzed within and among plant variation of spur length, flower length and internode length, and after taking account for effect being an early or late produced organ, spur length exhibited more within-plant variation than either of the other organs. Plants grow in a modular fashion which results in within plant variation of organs such as flowers, seeds and leaves (Herrera 2017). The larger variation is within a trait, the more likely that variation has ecological significance and can influence reproductive success (Herrera 2017). Differences in seed size and fruit set has been associated with variability within floral organs (Herrera 2017). Therefore, the within plant variation of spur length in *H. deflexa* remains an interesting character to examine ecological interactions.

I also confirmed reports that spur length tends to decrease from upper early produced flowers to lower later produced flowers. Decrease in organ size from first to last flower is a fairly common occurrence, as production and maintenance of flowers is extremely energetically costly for plants (Diggle 1995; Galen 1999). This decline in organ size could be attributed to a decline in available resources later in the season or could be genetically regulated (Powell and Lenhard 2012; Krizek and Anderson 2013). A flower's position within the architecture of the plant can greatly affect the amount of resources that flower is allocated (Diggle 1995). Plants have the ability of indeterminate growth in organs such as roots and stems, and determinate growth in other organs such as leaves and petals (Powell and Lenhard 2012). Growth of all organs is linked to genetics where a plant like *H. deflexa* will never grow taller than a Maple tree (Powell and Lenhard 2012). However, the growth within a plant is also highly linked with environmental conditions (Powell and Lenhard 2012).

In *H. deflexa*, most plants showed a decrease in all of the measured early season spur length, flower length and internode length. This decrease in size could be attributed to a combination of genetic predisposition and reduction in environmental resources late in the season. However, the pattern of within-plant change in spur length was much more variable than in flower length and internode length. In several instances spur length actually increased from

early flowers to late flowers. This suggests that some plants are reacting differently to temporal changes by increasing or maintaining nectar spur length. The direction of within-plant variation in a trait like spur length, may itself be a character that is affected by genotype (Harder et al. 2019). Moreover, this phenotypic variation between flowers can be acted upon by natural selection possibly resulting in evolution over time (Harder et al. 2019).

Testing for self-compatibility (Goal 2)

Unexpectedly, results from hand pollinations show that selfing flowers were more likely to set fruit than outcrossed fruit. It is important to keep in mind that these manipulations are not necessarily a true representation of what is occurring in nature, but they do show that *H. deflexa* is capable of physiologically selfing when self-pollen is physically placed on the stigma. Because the hand pollinated flowers are inside pollinator exclusion bags, they are potentially pollen limited compared to natural conditions (Aizen and Harder 2007; Chautá-Mellizo et al. 2012). Similarly, the flowers are not exposed to competition between outcross and self-pollen as they are subjected to only one type of pollen (Lloyd 1987; Chautá-Mellizo et al. 2012). Although the fruit set of self-pollinated flowers was significantly higher than the outcross pollinated flowers, the average number of seeds produced in each flower was not different. This could indicate that regardless of where pollen is coming from, each flower allocates a similar amount of energy into producing seeds. However, the quality and size of the seeds within each treatment has not yet been examined. Perhaps the selfed seeds are non-viable due to inbreeding depression resulting in increased homozygosity (Chautá-Mellizo et al. 2012). Inbreeding depression can also lead to lower quality offspring with fewer smaller flowers (Charlesworth and Willis 2009; Chautá-Mellizo et al. 2012). I would recommend that future studies take place on examining the quality of seeds by growing the hand outcross and selfed seeds in laboratory conditions.

Comparing unmanipulated fruit set in bagged pollinator excluded and open pollinator included plants gives a more accurate representation what is occurring and shows that even without pollinators *H. deflexa* produces self-fruit. Here we can see that unmanipulated flowers with access to pollinators are producing a similar fruit set to those with no pollinator access and only the ability to reproduce autogamously. This indicates that *H. deflexa* does not rely on pollinators for reproduction and can be just as effective in producing fruit without access to pollinators. Again, this experiment does not allow for the quality of seeds to be tested between

pollinator excluded and pollinator access treatments. Therefore, more analysis needs to be conducted on the quality of offspring to examine the potential effects of inbreeding depression.

Some species of plants have evolved to be almost completely self-reliant for fertilization and do not rely on pollinators despite the consequences of inbreeding depression (Sicard and Lenhard 2011). Successful autogamous species tend to follow similar characteristics including small flowers, reduced temporal and physical distance between anthers and stigma (Sicard and Lenhard 2011). Selfing species also tend to have less attractive flowers than their outcrossing counterparts, this can involve reduction in scent, nectar, pollen and pigmentation (Sicard and Lenhard 2011; Duncan and Rausher 2020). Several of these traits are shared with *H. deflexa*, including small flower size and proximity of anthers to stigma. However, *H. deflexa* produces conspicuous nectar spurs which are associated with attracting pollinators for outcrossing (Whittall and Hodges 2007). Although these nectar spurs were thought to attract pollinators to bring in outcross pollen, *H. deflexa* does not seem to need pollinators to reproduce successfully.

Evaluating reliance on autogamy from first-to-last flowers (Goal 3)

Both fruit set and seeds per fruit in all treatments of *H. deflexa* declined throughout the season. Flowers produced later in the reproductive season are often resource-limited and can be allocated less energy than flowers produced earlier in the reproductive season by the same plant (Ashman and Baker 1992; Diggle 1995; Kliber and Eckert 2004). Not only do late produced flowers have fewer resources available, they also have to compete with older flowers which are much further along in their reproductive life cycle (Diggle 1995). Therefore, a plant may strategically abort, or invest less energy into late produced flowers (Diggle 1995).

On open-pollinated plants, the fruit set of flowers that are able to reproduce through autogamy, geitonogamy and outcrossing was higher than the fruit set of emasculated flowers that could only reproduce through geitonogamy or outcrossing. Clearly autogamous reproduction is important to *H. deflexa* and can improve the amount of fruit produced. However, the fact that emasculated flowers set fruit at least some of the time demonstrates that some pollinator-mediated fertilization must have occurred. Moreover, pollinators may be more attracted to flowers with anthers and could bring in more outcross pollen leading to more outcrossing (Schoen and Lloyd 1992; Nicolson 2011). However, there was no effect of pollinators on fruit set in *H. deflexa* indicating nectar spurs may not actually attract pollinators in this instance.

Although it may be tempting to estimate rates of autogamy by simply subtracting the seeds produced in the emasculated treatment to seeds produced in the handle treatment, this does not allow for true estimates to be made. In natural conditions there may be competition between outcross and self-pollen that cannot be examined within the emasculated flowers of my experiment (Schoen and Lloyd 1992). Therefore, the seeds from this experiment will have to be genetically analyzed in order to determine paternity, which will allow a better estimate of rates of autogamy, geitonogamy and outcrossing. However, it is important to note that these estimates are not true rates of selfing and outcrossing that occur under natural conditions (Schoen and Lloyd 1992). At the start of the season handled flowers that have access to autogamous reproduction produce more seeds than emasculated flowers without autogamous reproduction, however by the end of the season the handled flowers produce a similar number of seeds as the emasculated flowers. This decline in number of seeds per fruit could be from a combination of declining resources, and a higher number of seeds aborted due to self-pollination (Martin and Lee 1993). By aborting selfed seeds *H. deflexa* can invest more energy into heterozygous outcross seeds all while decreasing inbreeding depression (Collevatti et al. 2009).

I observed no effect of pollinators on fruit set both goal 2 and 3 of my results, and no effect of pollinators on number of seeds per fruit in goal 3. Although I predicted that pollinator access would increase reproductive success early on in the flowering season on flowers with long nectar spurs, that prediction was not supported. Perhaps pollinators are not a reliable source of pollen and *H. deflexa* can assure reproduction by relying on self-pollination (Aarssen 2000). Further, there is no difference in the number of seeds produced by intact flowers on plants with or without pollinators. This indicates that *H. deflexa* could be entire self-reliant as both the fruit set and number of seeds per fruit are unaffected by pollinators.

Future Directions

Halenia deflexa is undoubtedly a species with a mixed mating system which can rely on autogamous fertilization. In order to further investigate *H. deflexa* I suggest examining the quality of seeds produced autogamously versus through outcrossing, examining the pollen ovule ratio, and determine paternity of the seeds produced in goal 3 to estimate rates of autogamy and outcrossing.

Investigating Seed Quality

Although results showed conflicting results on the effect of pollinators and autogamous reproduction on the number of seeds produced, it is important to investigate if the quality of seeds is different between outcross and selfed seeds. Inbreeding can increase homozygosity, thereby reducing offspring resilience and fitness (Charlesworth and Charlesworth 1987). Thereby a plant can potentially allocate different amounts of resources into a selfed vs outcrossed seed leading to a large variation of seed size which could affect overall fitness (Vaughton and Ramsey 1997). Furthermore, growing the offspring of the plants will allow examination of traits such as plant height, number of flowers, survivorship and growth rate to compare both selfed offspring to outcross offspring, and seeds produced early on in the season to late in the season (Vaughton and Ramsey 1997; Sheridan and Karowe 2000; Teixeira et al. 2009). This allows an examination into effects inbreeding depression and homozygosity can have on quality of offspring.

Pollen Ovule Data

An important change that often co-occurs with a shift in mating system between selfing and outcrossing is a change in the relative investment into pollen and ovule production. It is expected that there is a difference in investment in male and female organs between outcrossing and selfing plant species (Cruden 1977; Cruden and Lyon 1985). The relative investment into reproductive structures can be estimated by a pollen-ovule ratio, or by the relative biomasses of male and female structures (Cruden 1977). With either measure, the ratio of male-female investment is expected to be higher in outcrossing species than in cleistogamous or selfing species (Cruden 1977). The higher investment into pollen in outcrossing species can be attributed to the potential for lost pollen in transport, where in selfing species no pollen is lost in transport because it can be directly placed its own stigma. Pollen-ovule ratios were developed to compare species reproductive investment and can act as a proxy for comparison of mating systems (Cruden 1977; Cruden and Lyon 1985). There is evidence that the ratio of male-female investment can vary between outcrossing and selfing flowers within a plant (Delesalle et al. 2008). I have measured anthers and counted ovules from *H. deflexa* flowers throughout the 2019 summer, which can be analyzed in the future to see if and how these structures change from the start of the season to the end of the season.

Genetic testing of offspring to quantify rates of self- and outcross-pollination

Further research will need to be conducted in order to estimate rates of autogamy, geitonogamy and outcrossing. I collected leaf tissues from all parental plants from experiments aimed at examining temporal changes in reliance on self- versus outcross pollination (goal 3). Genotyping parents using this leaf tissue, and genotyping leaf tissue that grows from seeds collected from these same experiments will allow us to determine paternity of the offspring. It is important to genetically determine paternity as this allows for more solid conclusions to be made about the seeds collected from emasculated flowers that could only reproduce through outcrossing and geitonogamy, to the handled flowers that could reproduce through autogamy, geitonogamy and outcrossing. The handles produced more fruit than the emasculates, but in order to determine true paternity genetic testing must be conducted.

In order to perform the paternity analysis, a reliable procedure to grow *H. deflexa* in the lab will need to be completed. Determining the paternity of those seeds will allow for estimated rates of autogamy, geitonogamy and outcrossing to be directly determined, and will allow a deeper investigation into *H. deflexa* mating system. Genetic tests will not necessarily allow true rates of selfing and outcrossing to be made because in natural conditions there is competition between outcross and self-pollen which cannot be factored into the emasculated flowers (Schoen and Lloyd 1992). Further, emasculating a flower removes the potential source of self-pollen (Schoen and Lloyd 1992). However, determining paternity of collected seeds from all experimental flowers will provide more in-depth information about *H. deflexa* mating system.

Conclusion

As a self-compatible plant, *H. deflexa* could be completely self-sufficient and may not rely on pollinators for outcrossing. The results indicate that *H. deflexa* has a mixed mating system that relies heavily on autogamy. However, more work will need to be conducted in order to evaluate if there are trade-offs of seed quality in autogamously produced seeds. Similarly, future work should focus on estimating rates of autogamy and outcrossing through genetic analysis of seeds collected in these experiments. A decline in nectar spur length throughout the season could be an interesting characteristic to explore phenotypic variation and natural selection within a plant, but does not seem to affect the overall mating system from first to last flower.

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