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**Prolonged stigma and flower lifespan in females of the gynodioecious plant *Geranium sylvaticum***

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## Graphical abstract



## Highlights

- In gynodioecious plants females need a reproductive advantage over hermaphrodites
- Differences in floral longevity between sexes can provide a reproductive advantage.
- Stigma and flower lifespan was examined in gynodioecious *Geranium sylvaticum*.
- Stigma and flower lifespan are almost twice as long in females as in hermaphrodites.

## ABSTRACT

In gynodioecious plants females need a reproductive advantage over hermaphrodites to be maintained in the same population. Generally, three main proximate causes for a female advantage are considered: inbreeding avoidance, different resource allocation patterns, and differences in ecological interactions. A mechanism potentially causing a female advantage that is rarely discussed is a difference in floral longevity between the genders. Females may have a longer stigma lifespan than hermaphrodites, which can affect pollination. Stigma and flower lifespan are rarely documented in gynodioecious species, although it is a common observation in dioecious species that female plants flower longer than males. Here we focus on the stigma and flower lifespan of

gynodioecious *Geranium sylvaticum*, which could potentially contribute to the female advantage observed in this and other closely related species. We measured the stigma and flower lifespan in unpollinated flowers of female, hermaphrodite and plants with an intermediate gender expression. Our results show that stigma lifespan is almost twice as long in females as in hermaphrodites and intermediate plants. Also flower lifespan is longest in females. We discuss the potential mechanisms through which an increased floral lifespan can lead to a female advantage despite the generally lower pollinator visitation rates observed in females by reviewing available studies. Our study shows that increased floral persistence in females can be an important aspect in the maintenance of females in gynodioecious plants and should be taken into account as a potential proximate cause for a female advantage.

**Keywords:** Inbreeding avoidance, female advantage, flower persistence, gynodioecy, pollen limitation, pollination.

## 1. INTRODUCTION

Most flowering plant species are hermaphroditic, with individual flowers having both the male and the female function (Yampolsky and Yampolsky, 1922; Silvertown and Charlesworth, 2001). If a new genotype arises that produces flowers that have lost one of these functions, a reproductive advantage over the hermaphrodites is needed to invade and maintain itself in the population (Charlesworth and Charlesworth, 1978). The most common example of such polymorphic breeding system, found in approximately 2% of angiosperm genera (Dufaj et al., 2014; Renner, 2014), are gynodioecious plants, where populations consist of a mixture of individuals with either hermaphrodite or female (i.e. male sterile or pistillate) flowers. To be maintained in the population, females that only contribute genes to the next generation through ovules, need to have a higher seed

output than the hermaphrodites. If male sterility is solely determined by nuclear genes, the seed output needs to be at least double that of the hermaphrodites (Charlesworth and Charlesworth, 1978). A smaller advantage is enough if male sterility is determined by nucleo-cytoplasmic hereditary elements (Bailey et al., 2003).

Most studies on gynodioecious plants have indeed showed that total reproductive output is higher in females than in hermaphrodites. Females usually have a higher seed output, seeds that germinate better and/or have offspring of a higher quality (reviewed in Shykoff et al., 2003; Dufay and Billard, 2012). Such female reproductive advantage has generally been attributed to three main, non-exclusive, proximal causes (Shykoff et al., 2003; Dufay and Billard, 2012). First, females may avoid inbreeding, since they obligatorily need a hermaphrodite for pollination. Evidence for potential inbreeding depression in gynodioecious plants has been found (reviewed in Dufay and Billard, 2012). However, many plants, including many gynodioecious ones, have evolved mechanisms to avoid self-pollination. Flowers can be dichogamous with intrafloral protandry as the most common pattern (Lloyd and Webb, 1986), where the male function is active first and the female function later, or flowers can have complete or partial self-incompatibility.

Second, females investing relatively less in male reproductive tissue or floral attraction may allocate more resources towards higher seed output (Caruso et al., 2003). In low resource conditions, hermaphrodites may not be able to allocate enough resources to the female function and it is expected that females then have a relatively greater reproductive advantage (Ashman, 2007). In at least 14 gynodioecious species female frequency tended to be higher in populations where resource availability was lower (Ashman, 2007; Cuevas and López, 2011).

And third, the sex-specific ecological interaction hypothesis suggests that females may achieve higher seed production due to differences in ecological interactions with enemies or mutualists (Ashman, 2007; Vega-Frutis et al., 2013). Theoretically, females could be more

attractive to pollinators if they offered more nectar than hermaphrodites, leading to higher pollination and fertilization rates. However, we are unaware of any study on gynodioecious plants showing pollination discrimination in favor of female plants. In fact, female flowers are almost always smaller than hermaphrodite flowers (reviewed in Delph, 1996 and Shykoff et al., 2003; e.g. Bai et al., 2011; Barr and Fishman, 2011; Cuevas and López, 2011; Griffin and Byers, 2012; Blank et al., 2014; Cuevas et al., 2014) and/or they produce less nectar (Varga et al., 2013) and pollen, leading to lower pollinator visitation rates (Delph, 1996; Ashman, 2000; Bai et al., 2011). On the other hand, the smaller size and number of flowers of female plants may also render them less attractive to enemies like florivores or seed herbivores, leading to a relatively higher seed output in females than hermaphrodites (Marshall and Ganders, 2001; Ashman, 2002; Asikainen and Mutikainen, 2005a; Collin and Shykoff, 2009; Clarke and Brody, 2015).

Whereas these three mechanisms leading to a female advantage have received a lot of attention in recent studies and reviews (Shykoff et al., 2003; Ashman, 2007; Dufay and Billard, 2012; Vega-Frutis et al., 2013), a mechanism that is rarely discussed is the potential difference in flower and stigma lifespan, which can affect pollination levels. In dioecious and monoecious plants female flowers generally remain open for a longer time than male flowers (Primack, 1985). For several protandrous gynodioecious plants it has been suggested that while overall flower lifespan may not differ between the sexes, female flowers become receptive earlier for pollen and have a longer stigma lifespan than hermaphrodite flowers (Shykoff, 1992; Williams et al., 2000; Rodriguez-Riano and Dafni, 2007; Molano-Flores and Faivre, 2015).

Only four studies have actually measured and compared floral lifespan in gynodioecious plants and all showed a longer floral lifespan in females. However, just one study compared floral lifespan between hermaphrodite and female flowers in the absence of pollinators (Arnan et al., 2014). In the other three studies (Ashman and Stanton, 1991; Pettersson, 1992; Yao and Luo, 2011) all flowers were allowed to be naturally pollinated and early floral or stigma senescence in the

hermaphrodites may have been induced by faster pollination due to higher insect visitation, as floral persistence may depend on pollination (e.g. Niu et al., 2011).

Here we focus on the stigma and flower lifespan of gynodioecious *Geranium sylvaticum* (Geraniaceae) as a potential contributing factor to the female advantage observed in this and other closely related *Geranium* species (Table 1). *Geranium sylvaticum* is assumed to have a mixed nuclear-cytoplasmic sex determination system (Asikainen, 2004). The female frequency varies considerably between populations and ranges from 0 to 27% with an average of about 15% (Vaarama and Jääskeläinen, 1967; Asikainen and Mutikainen, 2003; Volkova et al., 2007). Female flowers are markedly smaller and have lower nectar sugar content than hermaphrodite flowers (Table 1). Also, insect visitation rates have been found to be lower for females (Table 1), although visitation rate by bumblebees may not differ between the sexes (Varga and Kytöviita, 2010). According to Asikainen and Mutikainen (2005a) pollen limitation is generally quite low and there was no difference in pollen limitation between sexes in this species. Hermaphrodite flowers are protandrous with the stigmatic lobes closely joined during initial pollen presentation (Varga et al., 2013), believed to strongly reduce autogamy. Female flowers start unfolding their stigma early after flower opening (Varga et al., 2013). Individual flower lifespan has not been measured, but for the closely related *G. richardsonii* it was suggested, but not measured, that female flowers have 2-3 days longer lifespan than hermaphrodites (Williams et al., 2000). Some *G. sylvaticum* plants produce female and hermaphrodite flowers, or flowers with a reduced number of anthers (flowers of intermediate sexual expression) (Putrament, 1962). The reported frequency of intermediates in populations ranges from 0 to 43% (Putrament, 1962; Volkova et al., 2007).

In this study, we tested whether the maximum flower and stigma lifespan in the absence of pollination is longer in females than in hermaphrodites of *G. sylvaticum*. We monitored individual flowers and recorded how long the flower and stigma remained open and receptive. A variable number of functional anthers in intermediate plants allowed us to test whether the level of male

gender expression is correlated with flower and stigma lifespan. We discuss the potential mechanisms through which increased stigma lifespan can lead to a female advantage in this and other gynodioecious species.

## 2. MATERIALS AND METHODS

### 2.1 Plant species

Wood cranesbill, *Geranium sylvaticum* L. is a very common perennial herb that grows from heavily shadowed to non-shadowed habitats like deciduous forests, moist meadows and roadsides in large parts of Eurasia. In Central Finland flowering starts at the end of May with a peak in June and usually ends in July. According to Asikainen and Mutikainen (2005a), female plants start flowering three days earlier than hermaphrodites. The protandrous hermaphrodite flowers produce 10 stamens (Fig. 1A) with the anthers presenting pollen shortly after flower opening (Varga et al., 2013). For a variable period of time during this male phase, the five lobes of the stigma remain closely joined, after which the female phase starts when the lobes curl outwards thus exposing the pollen receptive surface (Fig. 2). In naturally pollinated, unmanipulated plants, the stigma unfolding occurs frequently after all the anthers have dropped (Müller, 1881). In females (Fig. 1B) the stigma generally opens during or shortly after flower opening. Nectar is produced at the base of the flower (Varga et al., 2015). Flower visitors and potential pollinators include bumblebees and other hymenopterans, syrphid flies and other diptera, and a pre-dispersal seed predator specialist weevil *Zacladus geranii* (Varga and Kytöviita, 2010; Varga, 2014). When the flower senesces, the petals drop, usually within a short period of time or all at once due to a disturbance (e.g. wind or a bumblebee visit, pers. obs.). Whereas Müller (1881) anecdotally reports a few observations of nectar consuming insect visitors on petal free flowers, Willson et al. (1979) did not observe any visitors after all petals were abscised in *Geranium maculatum*. Flowers have 10 ovules but usually a maximum of five seeds ripen per flower.

Most populations are gynodioecious with female plants producing male sterile flowers that have degenerated stamens and do not produce functional pollen (Fig. 1C). Stamen degeneration ranges from flowers having fully developed stamens but with the anther only producing sterile deformed pollen, through degenerated anthers to missing anthers and rudimentary filaments. Also, in many populations plants with intermediate flowers can be found that have a reduced number of 1-9 functional anthers (Putrament, 1962; Volkova et al., 2007). The level of male sterility in intermediates usually varies per flower within individual plants.

## **2.2 Experimental conditions**

Flowering plants from natural populations around Jyväskylä, Finland, were dug out at the start of the flowering season (end of May) and replanted into 1.5 L pots. We selected plants based on the first open flowers, being either female or hermaphrodites, although several individuals developed intermediate flowers during the experiments. All plants were allowed to recover for approximately one week before flower monitoring was commenced and watered when necessary. Floral lifespan of female plants (defined as having on average less than 1 functional anther per flower), full hermaphrodite plants (on average more than 9 anthers per flower) and intermediate plants (on average 1-9 anthers per flower) was measured in three experiments with differing environmental conditions that each lasted about one week, using separate sets of plants.

In 2013 we performed two greenhouse experiments with different plants under different temperature regimes, with temperatures ranging from 21-30°C (Experiment 1) and 21-23°C (Experiment 2). In 2014, with newly collected plants, we performed an outdoor experiment (Experiment 3) under more natural weather conditions in a large meshed cage that kept pollinators out but allowed light, rain and wind to get in, with temperatures ranging from 10-29°C. Here we also explored the effect of anther removal on floral lifespan and compared the effect of pollen deposition between females and the male and female phase in hermaphrodites.

### 2.3 Measurements

In each experiment, plants were checked for newly opened flowers at intervals of 1-10 hours for three days. Flowers opening during this period were followed over time and checked for a change in flower status every 1-10 hours (average interval between checks was 2.3 hours), i.e. we noted the time the flower opened, the stigma opened, and when the flower had lost 1, 2, 3, 4 or all 5 petals.

The number of functional stamens (anthers with visible pollen) was counted in all experimental flowers. The flower size was estimated by measuring the length of one petal (from the base to the tip) in all flowers in Experiment 1 and 2, and from one random flower per plant in Experiment 3 (since measurements in Experiment 1 and 2 showed that flower size was quite consistent within plants).

To examine whether pollen removal would affect floral lifespan, in Experiment 3 we removed the anthers immediately after petal opening in approximately half of the full hermaphrodite flowers. To gain more information about stigma receptivity, we hand-pollinated 45 random female flowers with long-lasting stigmas (stigma lifespan longer than two days). Similarly, 46 hermaphrodite flowers with recently opened stigmas (up to one day) and 45 more hermaphrodite flowers with closed stigmas (Fig. 2A) were hand-pollinated to confirm that during that stage stigma receptivity is negligible. In all cases, we first attached a large amount of pollen to a small paintbrush by touching anthers from several flowers within the experiment but not used in the observations. Subsequently, we touched the whole exposed surface of the style with the brush. All flowers used in the experiments, including the non-pollinated, were later examined for the development of seeds to check for fertilization.

### 2.4 Data analyses

We calculated flower lifespan as the time between the last status check before flower opening and the first status check when petals were dropped. We assume that flower attractiveness to pollinators effectively ceases when all petals have dropped. Still, it is unknown how many petals are needed for flowers to remain attractive to pollinators. Therefore, we calculated five flower lifespan values for each flower corresponding to the time until each petal (1-5) had dropped. Similarly, we calculated stigma lifespan using the time of the last status check before the stigma opened as starting point until each petal had dropped. Because using the lifespan measures until 2, 3 or 4 petals dropped did not give a qualitative difference in statistical analyses, only the results of the lifespan measures until the first or all five petals dropped are presented.

To test whether floral lifespan and petal size were different between plant genders we used linear mixed models, with gender (female, intermediate, hermaphrodite) and experiment (1, 2, 3) and their interaction as fixed variables and plant nested in the gender  $\times$  experiment interaction as random variable. If the interaction or the experiment were not significant ( $P > 0.05$ ) they were removed from the models. Significant effects for gender were further investigated by pairwise analyses for each gender pair. For the data on hermaphrodites in Experiment 3, we used a linear mixed model with plant as a random factor to test for the fixed effect of cutting anthers on floral lifespan.

To test whether petal size and floral lifespan were correlated, we used the average flower values per plant. Subsequently an ANCOVA was used with petal size as a covariate and (if significant) the interaction with gender, analyzing the residuals of the mean per gender group (female, intermediate, hermaphrodite).

For the intermediate plants only, we tested if the number of anthers correlated with floral lifespan with Pearson correlation and with an ANCOVA with experiment as a factor and number of anthers as a covariate. To test for a correlation between the number of anthers and flower size in the

intermediate plants, without taking into account the potential effect of plant, we used an ANCOVA with experiment as factor.

All lifespan and petal size data were square-root transformed before analyses to acquire normal distributions in the residuals. Also, a few cases where stigma lifespan values were 0 (i.e. the stigma opened after petals had dropped) were excluded from the presented analyses as they prevented the normal distribution of the residuals. Including these data did however not qualitatively change the outcome of statistical tests.

We used pairwise chi-square and t-tests to test for differences in fruit and seed set between the three hand-pollinated groups. All analyses were performed in IBM SPSS 22 (IBM Corp., Armonk, NY, USA).

### **3. RESULTS**

#### **3.1 Floral and stigma lifespan**

In total, we measured floral lifespan on 122 female, 129 hermaphrodite and 111 intermediate flowers on, respectively, 21, 14 and 13 plants. Flowers from female plants had significantly longer stigma lifespan than flowers on both intermediate and hermaphrodite plants (Table 2, Fig. 3A), but no significant difference between intermediates and hermaphrodites was observed (Table 2). Based on plant means, it took on average 66.3 hours on female plants from stigma opening until the first petal dropped whereas on male functional plants (intermediate and hermaphrodites) it was 31.3 hours (Fig. 3A). In nine (out of 120) flowers on hermaphrodites the stigma did not even open before all petals had dropped (see Fig. S1).

Although the difference was much smaller for flower lifespan, flowers on female plants remained open for a significantly longer time than flowers on intermediate and hermaphrodite plants (Table 2, Fig. 3B). Again, no difference was found between intermediate and hermaphrodite plants (Table 2). Based on plant means, flower lifespan until the first petal dropped was on average

73.1 hours on female plants, whereas on male functional plants (intermediates plus hermaphrodites) it was 56.2 hours (Fig. 3B). There was also an effect of experiment on floral lifespan, if measured until the first petal dropped (Table 2). This was due to flowers in experiment 1 having on average a shorter floral lifespan than in experiment 2 (stigma:  $P_{\text{experiment}} = 0.03$ , flower:  $P_{\text{experiment}} = 0.03$  pairwise linear mixed models). If only data from the 2014 experiment, where flowers were exposed to wind, were analyzed, flower lifespan was not significantly different between the genders (linear mixed models,  $P_{\text{gender}}=0.52$  and  $P_{\text{gender}}=0.39$  for flower lifespan until the first and last petal dropped, respectively). In the greenhouse experiments, variation between plants in flower lifespan was larger than in the experiment in the outside cage (Fig. 3), perhaps due to the absence of wind.

Cutting anthers at the onset of flowering did not statistically affect flower or stigma lifespan (linear mixed models,  $F_{1,69.3} = 1.209$ ,  $P = 0.28$  and  $F_{1,69.3} = 0.010$ ,  $P = 0.92$  for stigma and flower lifespan, respectively, up to first dropped petal) in the hermaphrodite flowers. In the intermediate plants, the average number of functional anthers per plant was not correlated with stigma or flower lifespan (all  $P_{\text{covariate}} > 0.05$ , Fig. S2)

### 3.2 Petal size

The length of the petals differed significantly among all the three genders (Table 2); female plants had the smallest flowers, hermaphrodites the largest and intermediates had flowers of intermediate size (averages of plant means =  $9.7 \pm 0.4$ ,  $13.1 \pm 0.5$  and  $14.7 \pm 0.7$  mm for females, intermediates and hermaphrodites, respectively). Within each gender group, average petal size was not correlated with any average lifespan measurement (all  $P_{\text{covariate}} > 0.05$ , Fig. 4, Supporting Information Fig. S3). For the intermediate plants, there was no correlation between average petal size and average stigma or flower lifespan (all  $P_{\text{covariate}} > 0.05$ ). On a per flower basis (not accounting for a potential plant effect) we found a statistically positive correlation in the intermediate plants between anther number and petal size (ANCOVA,  $F_{1,55} = 20.024$ ,  $P < 0.01$ ), but if the few fully female and fully hermaphrodite flowers were excluded there was no remaining significant correlation.

### 3.3 Fruit and seed set

None of the non-pollinated flowers produced seeds, suggesting that selfing through geitonogamy or autogamy does not occur in the absence of insects. For the hand-pollinated flowers, only 1 out of the 45 hermaphrodite flowers with closed stigma produced seeds, which was significantly less than the 28 out of 46 flowers with stigma recently opened ( $X^2_1 = 36.0$ ,  $P < 0.01$ ) or the 21 out of 45 female flowers with long lasting stigmas ( $X^2_1 = 24.1$ ,  $P < 0.01$ ). The fertilization success in the two-day old female flowers (46%) was somewhat lower than for the young hermaphrodite flowers (61%) but not significantly different ( $X^2_1 = 1.85$ ,  $P = 0.17$ ). The oldest hand-pollinated female stigma for which age at pollination is known that produced seeds, had a stigma that was 72 hours old at pollination. The average number of seeds produced by fertilized females was significantly lower than for the young hermaphrodite flowers ( $2.90 \pm 0.26$  vs.  $3.82 \pm 0.22$  s.e. respectively,  $t = 2.681$ ,  $P = 0.01$ ).

## 4. DISCUSSION

This study shows that maximum stigma and flower lifespan is longer in female flowers than in hermaphrodite flowers in unpollinated gynodioecious *G. sylvaticum*. Stigma lifespan was on average more than twice as long in female flowers as in hermaphrodite ones. In the absence of pollinators, hermaphrodite flowers remained receptive for pollen for a bit longer than one full day, whereas female flowers remained open for about three days while being in the pollen-receptive phase. This was mainly due to the early opening of the stigma in female flowers compared to the protandrous hermaphrodite flowers, but also due to the increased total flower lifespan in females.

In the only previous study where stigma lifespan of gynodioecious unpollinated flowers was measured, stigma lifespan was about one day longer in female flowers compared to hermaphrodites (*Thymus vulgaris*, Arnán et al., 2014). This difference was similar for naturally pollinated flowers,

except that those flowered four days shorter. Other studies on naturally pollinated flowers also showed an extended period of stigma lifespan for females (Ashman and Stanton, 1991; Petterson, 1992), and it has been suggested (but not measured) for several other species, including *Geranium* (Shykoff, 1992; Williams et al., 2000; Rodriguez-Riano and Dafni, 2007; Molano-Flores and Faivre, 2015). Whereas these other studies may indicate that extended maximum stigma lifespan in females is a general phenomenon among protandrous gynodioecious species, the differences in stigma lifespan may also be explained by the higher visitation rates observed for hermaphrodites. It is well known that pollination and fertilization can induce wilting of the stigma and flower, also in gynodioecious plants (Van Doorn, 1997; Niu et al., 2011; Yao and Luo, 2011). Whether senescence is induced in *G. sylvaticum* by pollination is unknown, but it is likely as it has been described or suggested for other species in this genus like *G. pyrenaicum* (Fitting, 1911), *G. pusillum* (Schulz, 1902) and *G. maculatum* (Van Etten and Chang, 2014). Also, *G. thunbergii* hermaphrodite flowers that were artificially or naturally pollinated flowered on average about one day shorter than unpollinated hermaphrodite flowers (Kandori, 2002).

Female *Geranium* plants are in general less attractive to potential pollinators than hermaphrodites (Table 1), possibly due to their smaller flowers, reduced production of nectar or the absence of pollen. Asikainen and Mutikainen (2005b) followed individual syrphids and bumblebees and estimated that on average pollinators preferred hermaphrodites 13% more than females. Varga and Kytöviita (2010) observed that experimental hermaphrodite plants were visited 1.5 times as much as females during 30 minutes trials. However, when bumblebee visits (23% of all visits), which are potentially the most important pollinator group of *Geranium* (Kandori, 2002) were analyzed separately, no clear preference for either sex was detected. Higher visitation rates for hermaphrodites have been reported also in other *Geranium* species (Table 1). However, no data are available for the total number of visits that flowers of the two genders may receive before wilting. Also, importantly, in these studies it was not recorded whether the stigma of the hermaphrodite

flowers had already opened. It is possible that visitation rate decreases after the male phase, because nectar production declines in the female phase (Varga et al., 2013) and most pollen, which is attractive to pollen collecting species, may have been removed by previous visitors (Klinkhamer and de Jong, 1990).

A longer stigma lifespan in females can potentially compensate for reduced pollinator attractiveness and may even lead to a female advantage through several mechanisms. First, an extended stigma lifespan should increase the probability of being visited by a pollinator. This can be especially relevant when a single visit can already lead to fertilization and fruit set and pollinators are scarce. If stigma lifespan in females is larger than in hermaphrodites, the total number of visitors to female flowers may be equal or longer than for hermaphrodites, depending on the relative increase in female stigma lifespan. This could subsequently lead to equal or larger amounts of pollen deposited on females, despite their lower visitation rates. In some plants fertilization and fruit set can only take place after a threshold number of pollen grains are deposited on the stigma (Delph and Havens, 1998) and extended stigma lifespan may thus increase the probability of receiving sufficient pollinator visits for fruit set before flower wilting. In several studies on *G. sylvaticum* and *G. maculatum* an increased fruit set for females has been observed (Table 1), thus their extended stigma lifespan could have contributed.

Second, a larger number of pollinator visits usually leads to higher pollen deposition, as observed in *G. maculatum* (Van Etten and Chang, 2014). This can subsequently result in more pollen tubes, more fertilization and ultimately a higher seed set per fruit. In fact, Van Etten and Chang (2014) reported a positive correlation between the number of pollen grains deposited and seed set per flower. In our study, stigma lifespan was on average about twice as long in females as in hermaphrodites. If visitation rate would be constant over time, a female would receive the same amount of visits with only half the hermaphrodite visitation rate. Since both Varga and Kytöviita (2010) and Asikainen and Mutikainen (2005a) found on average a much smaller difference in

visitation rate (respectively, a 13% or 50% higher visitation rate in hermaphrodites), total pollen deposition under natural circumstances could potentially be higher in females, leading to less pollen limitation and higher seed set per fruit. Unfortunately natural pollen deposition numbers are not available for *G. sylvaticum*. For *G. maculatum* and *G. richardsonii*, total pollen deposition on female flowers is on average lower than on hermaphrodites (Table 1). For other gynodioecious plants, results on pollen deposition are mixed. Some studies show similar pollen deposition in both genders (Ashman and Stanton, 1991; Shykoff, 1992) while others observed higher pollen loads on female flowers (Bai et al., 2011) or on hermaphrodites flowers (Alonso et al., 2007; Orellana et al., 2005).

A third mechanism by which stigma longevity may contribute to a female advantage is a higher number of pollen grains (potentially acquired through an increased total visitation) leads to pollen competition and a resulting stronger male gametophyte selection process and better quality offspring (Delph and Havens, 1998). Shykoff (1992) reported that female flowers in *Silene acaulis* had a higher number of pollen tubes than hermaphrodites, presumably because they became more early receptive and received more pollen at an early age. Since the number of pollen tubes was outnumbering the number of ovules this provided a competitive arena for the male gametophytes. Indeed, offspring of female flowers had a higher survival rate than those of outcrossed hermaphrodite flowers (Shykoff, 1992). In *G. sylvaticum* only two studies have looked at germination rates and did not find a higher quality seeds for females (Table 1), but for *G. maculatum* both germination rate and seedling size were larger for seeds from female flowers (Chang, 2006). Since deposited pollen probably outnumbers the maximum of five seeds per fruit in *G. sylvaticum* under natural pollination conditions, pollen competition may play a role in this species.

Not only stigma lifespan but also flower lifespan was somewhat greater in our female plants. Although it is well known that female flowers have increased persistence compared to males in

dioecious and monoecious plants (Primack, 1985), only one study has reported flower persistence in a gynodioecious species. For open-pollinated *Potentilla tanacetifolia* female flowers remained open for several hours longer than hermaphrodite flowers (Yao and Luo, 2011), but whether this also translated into increased stigma lifespan was not measured. Increased flower lifespan in females may cause an increase in total flower display if individual flower phenologies overlap, as is the case also in *G. sylvaticum*. Indeed, Yao and Luo (2011) found that in the late afternoon, most female plants still presented a large flower display whereas hermaphrodites had only small numbers of flowers open. This could potentially lead to an increase in visitor attractiveness to the plant. For *G. sylvaticum*, however, no significant correlation was observed between the number of flowers on a plant and the number of visits received during a 30 min interval (Varga and Kytöviita, 2010). Our data suggest that under natural circumstances, i.e. with wind present to initiate petal dropping, flower lifespan in females may not be higher than the other genders.

In our study, all floral visitors were excluded. Whether the gender specific differences in floral lifespan will be observed on natural, insect visited plants, as has been suggested for *G. richardsonii* (Williams et al., 2000), depends on whether flower visitation and/or pollination have an effect on the duration of the floral phases. Pollinators cannot only induce senescence, shortening the female phase, but by removal of pollen they can also induce the female phase in some protandrous species (Evanhoe and Galloway, 2002; Zhang et al., 2011). In our experiment we did not see such an effect after artificial anther removal. If removal of pollen or pollination affects the length of the female phase, floral and stigma persistence of each gender will depend on the (relative) visitation rates and may thus be strongly dependent on local and temporal variation in the pollinator density. Even within habitats local variances in conditions such as shading may have large effects on pollinator visitation rates and therefore potentially on the relative and absolute reproductive advantage of females.

The maintenance of flowers and stigmas likely requires resources from the plant, such as water and nutrients (Primack, 1985). Since female flowers are smaller we expected them to increase floral lifespan (Ashman and Schoen, 1996). However, we failed to find a correlation between flower size and floral lifespan within the two genders. We did not measure stigma sizes, but sexes had similar pistil weight in *G. sylvaticum* (Ramula and Mutikainen, 2003). Interestingly, intermediate plants with a reduced level of male functionality had no different flower or stigma lifespan than fully hermaphrodite plants even though their petal size is also intermediate. This suggests that floral lifespan is not strongly dependent on resources, at least not with ample water and nutrient supply, and is only prolonged in the case of full male sterility. Flower size on the other hand seems to be determined more by the level of male functionality, possibly due to a strong developmental correlation between the stamens and petals, because of a genetic linkage or pleiotropic effects (Raab and Koning, 1988; Schultz, 2003; Barr and Fishman, 2011), even though we did not find a correlation between the number of functional anthers and flower size within the intermediates.

## 5. CONCLUSIONS

Our study shows that in the gynodioecious plant *G. sylvaticum* flower and stigma lifespan is longer in females than in hermaphrodites in the absence of pollinators. This may potentially contribute to the female advantage reported for several traits (larger fruit set, larger seed set per fruit, higher quality of seeds) in several studies on this and other, closely related species. Inbreeding and selfing avoidance, resource allocation towards female reproduction and a reduction in attractiveness to enemies are clearly important proximate causes of female advantage in many gynodioecious plant species (Dufay and Billard, 2012). Nevertheless, we suggest that more attention should be paid to potential differences in flower and stigma persistence as it may contribute to higher fruit set, seed set and seed quality in females, despite their smaller attractiveness to pollinators.

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**FIGURE LEGENDS:**

**FIGURE 1.** A) Hermaphrodite, B) female, and C) intermediate flowers of *Geranium sylvaticum*.

Photos by Sandra Varga.

**FIGURE 2.** Three stages of the style of a protandrous hermaphrodite *Geranium sylvaticum* flower.

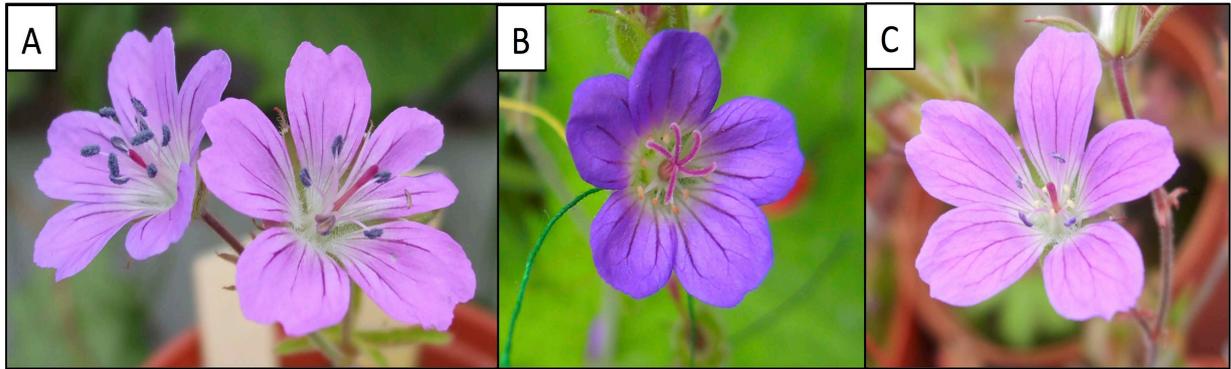
A) During the male phase the stigmatic lobes remain tightly closed. B) The stigma opens at the top.

C) The lobes curl outwards and the stigmatic receptive surface becomes fully exposed. Photos by Jelmer Elzinga.

**FIGURE 3.** Average ( $\pm$  s.e.) stigma (A) and flower (B) lifespan (in hours) for female, intermediate and hermaphrodite *Geranium sylvaticum* plants in the absence of pollinators. Dashed lines indicate the estimated marginal means from a linear mixed model for each sex expression group.

**FIGURE 4.** Average flower lifespan  $\pm$  s.e. (from flower opening until the first petal dropped, in hours) plotted against average petal length in female, intermediate and hermaphrodite plants.

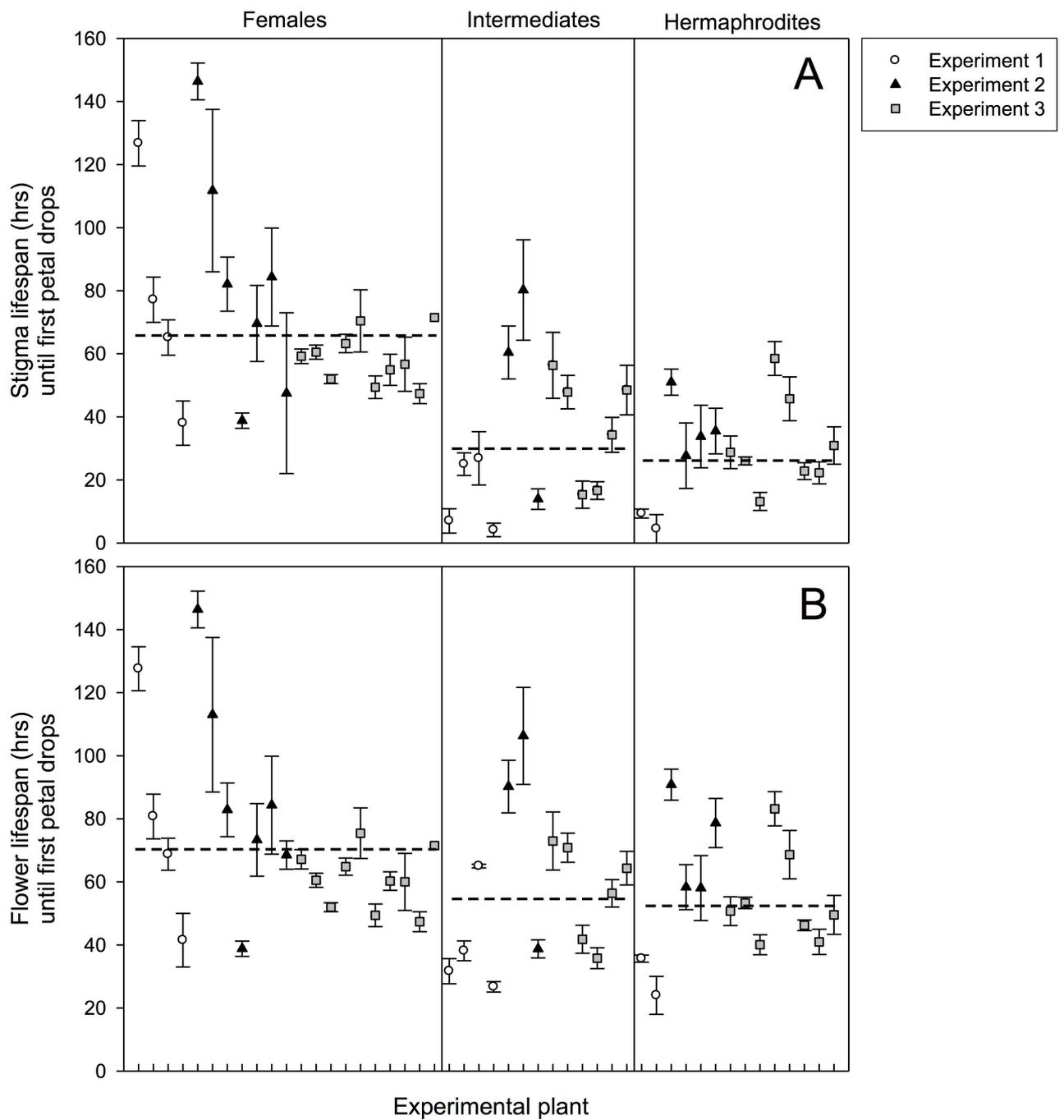
**FIGURE 1.** A) Hermaphrodite, B) female, and C) intermediate flowers of *Geranium sylvaticum*. Photos by Sandra Varga.



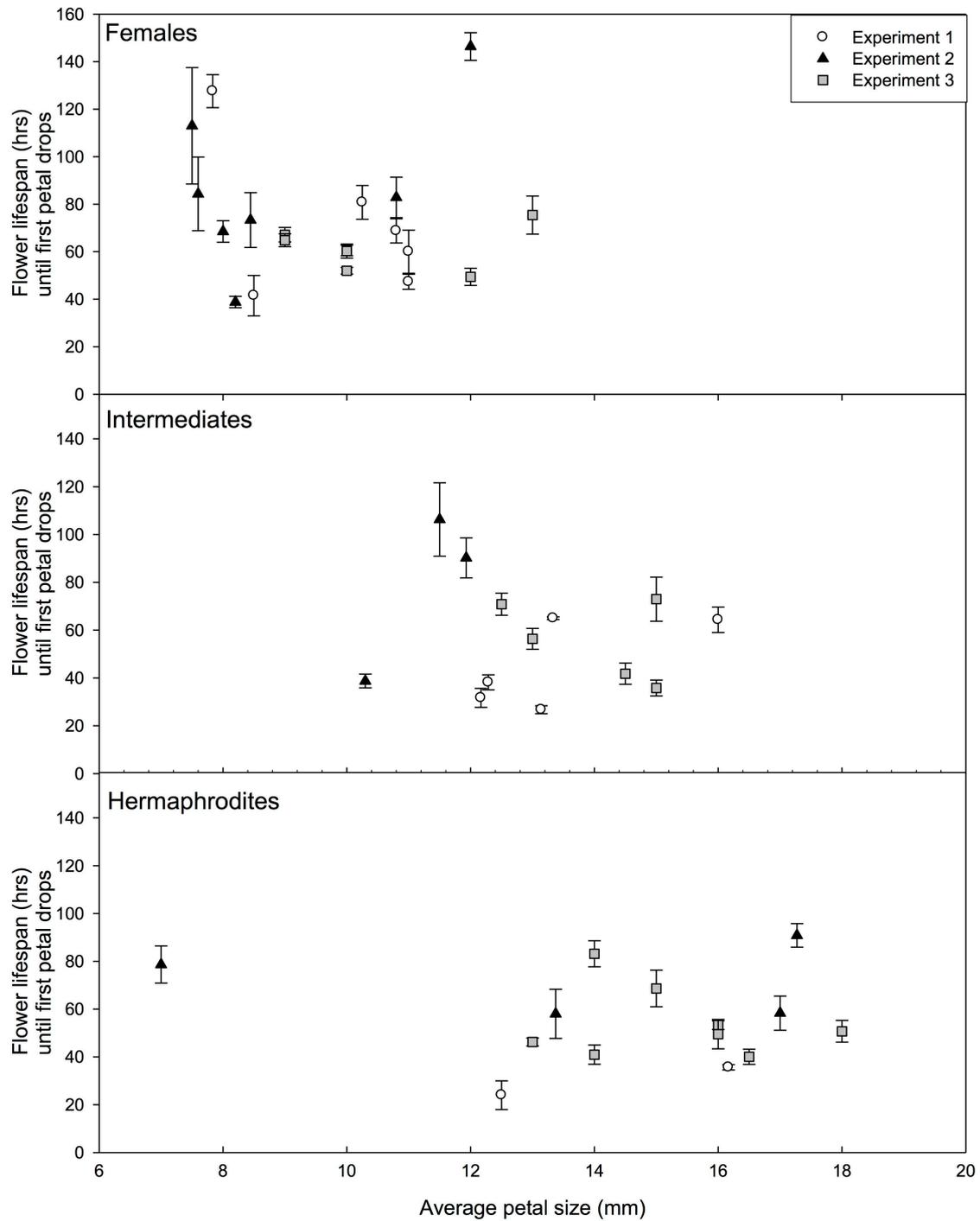
**FIGURE 2.** Three stages of the style of a protandrous hermaphrodite *Geranium sylvaticum* flower. A) During the male phase the stigmatic lobes remain tightly closed. B) The stigma opens at the top. C) The lobes curl outwards and the stigmatic receptive surface becomes fully exposed. Photos by Jelmer Elzinga.



**FIGURE 3.** Average ( $\pm$  s.e.) stigma (A) and flower (B) lifespan for female, intermediate and hermaphrodite *Geranium sylvaticum* plants in the absence of pollinators. Dashed lines indicate the estimated marginal means from a linear mixed model for each sex expression group.



**FIGURE 4.** Average flower lifespan  $\pm$  s.e. (from flower opening until the first petal dropped) plotted against the average petal length in female, intermediate and hermaphrodite plants.



**TABLE 1.** Summary of studies examining differences in reproductive characters between females and hermaphrodites in *Geranium* species. If the studies were performed on multiple populations and no overall statistical test was presented, we show the effect observed in the majority of populations. We only show results from published data, discarding personal observations or unpublished data that were mentioned but not

Species	Location	Pollination	Reproductive morphology				Pollination			Seed Production				Reference	
			Bud number	Flower number	Flower size	Pistil mass	Nectar volum	Visitation rate	Pollen deposition	Fruit set	Total fruits	Seeds/ flower	Seeds/ plant		Seed size
<i>G. sylvaticum</i>	Natural	Supplemental								F > H	F > H	F > H	F = H		Asikainen and Mutikainen, 2005b
	Natural	Natural								F > H	F > H		F = H		Asikainen and Mutikainen, 2003
	Natural	Natural								F > H	F > H	F > H			Asikainen and Mutikainen, 2005b
	Natural	Natural		F < H				F < H						F = H	Asikainen and Mutikainen, 2005a
	Natural	Natural	F = H			F = H					F = H	F > H	F = H		Ramula and Mutikainen, 2003
	Natural	Natural		F < H	F < H						F = H	F < H	F < H	F = H	Vaarama and Jääskeläinen, 1967
	Natural	Natural	F = H	F < H						F = H	F = H	F = H	F = H		Varga, 2014
	Garden	Natural		F = H	F < H			F < H		F > H		F > H			Varga and Kytöviita, 2010
	Garden	Natural	F = H							F > H	F > H	F > H			Varga et al., 2009
Garden	-						F < H*								Varga et al., 2013
<i>G. maculatum</i>	Natural	Natural	F = H	F < H						F > H	F > H		F = H		Agren and Willson, 1991
	Natural	Natural	F = H	F < H						F = H	F > H	F > H	F > H	F > H	Chang, 2006
	Garden	Natural	F < H	F < H		F = H	F < H	F < H			F = H				Van Etten and Chang, 2014
	Greenhouse	Artificial	F > H	F < H						F = H		F = H	F = H		Van Etten et al., 2008
<i>G. richardsonii</i>	Natural	Natural		F < H			F < H	F < H							Williams et al., 2000

presented.

Note:\* Nectar sugar content

**TABLE 2.** Differences between plant genders (females, hermaphrodites and intermediates) in floral lifespan and petal size. Statistical results and estimated marginal means from linear mixed models with gender and experiment as fixed factors and plant (nested in gender × experiment) as a random factor. Non-significant (N.S.) gender × experiment interaction or experiment effects were removed from the models. Estimated means with different letters were significantly different ( $P < 0.05$ ) from each other according to pairwise linear mixed models.

	Gender (N=3)			Experiment (N=3)			Estimated marginal means for gender*		
	df	<i>F</i>	<i>P</i>	df	<i>F</i>	<i>P</i>	Females	Intermediates	Hermaphrodites
Stigma lifespan (hours)									
Until first dropped petal	41.776	19.852	<0.001	43.634	4.564	0.016	66.32 ± 0.15 a	30.39 ± 0.21 b	27.23 ± 0.22 b
Until last (5 <sup>th</sup> ) dropped petal	43.164	17.025	<0.001	N.S.			79.55 ± 0.15 a	36.20 ± 0.22 b	38.88 ± 0.22 b
Flower lifespan (hours)									
Until first dropped petal	42.162	3.495	0.039	43.386	5.173	0.010	70.17 ± 0.09 a	54.82 ± 0.14 b	53.33 ± 0.14 b
Until last (5 <sup>th</sup> ) dropped petal	43.763	3.769	0.031	N.S.			82.77 ± 0.10 a	62.71 ± 0.15 b	63.55 ± 0.14 b
Petal size (mm)	36.716	29.701	<0.001	36.725	4.084	0.025	9.63 ± 0.01 a	12.71 ± 0.01 b	14.62 ± 0.01 c

Note:\* back-transformed values