

Pollen thieving and pollen limitation in gynodioecious *Polemonium foliosissimum*
(Polemoniaceae)

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Abstract

The limitation of pollen available to pollinators and visitors alike may potentially direct pollinator visitation choice as well as the plant's ability to provide pollen rewards, nectar rewards, and plant defense mechanisms. Such trade-offs exist in *Polemonium foliosissimum* A. Gray (Polemoniaceae) a gynodioecious perennial plant in that we tested the hypothesis that pollen limitation would correlate to differences in stigma pollen deposition and plant fitness between females and hermaphrodites within a population sampled off of Kebler Pass Road near Crested Butte, CO (38.86301,-107.12087). As theory dictates that females must either produce more seeds, seeds of higher quality, or render themselves less attractive to herbivores and pre-dispersal seed predators to compensate for loss of male fitness in order to persist in gynodioecious populations (Burd 1994). The role of trade-offs effectively can alter plants' choices of resource allocation between attracting pollinators and limiting harm from enemies. As such this balance has been investigated for herbivores, florivores, and pre-dispersal seed predators such as *Hylemya (Delia)* sp. (Diptera: Anthomyiidae), yet there has been insufficient attention in regards to these trade-offs in response to pollen thieves.

Pollen thieving muscid flies (Diptera: Muscidae) consume protein-rich pollen with little to no direct pollination. Within hermaphroditic and female *P. foliosissimum* hermaphroditic advantages of offering protein-rich pollen and male siring capabilities are essentially lost to pollen thieves. This therefore allots females representative equal fitness amongst the gynodioecious population and can potentially warrant correlation with differences in fitness via seed-set quality and quantity between *P. foliosissimum*. Nevertheless, throughout the manipulation of pollen neighborhoods by removing half of the available pollen from *Polemonium* in ten 2m² plots we saw significant differences in pollen deposition between hermaphroditic and female flowers ($p < 0.001$), but not between manipulation treatments, as denoted as "pre-treatment" and "post- treatment" ($p = 0.3892$) Pollinator visitation rate as well substantiated that pollinators and pollen thieving muscid flies on average spend 95% more time upon hermaphroditic flowers as compared to female flowers ($p < 0.001$). Yet, pollinators and enemies significantly visit more hermaphrodites than females on average ($p < 0.001$), but do not significantly discriminate between number of flowers visited between hermaphrodites and females ($p = 0.0539$) as analyzed in a One-way ANOVA using JMP.

Introduction

Plants allocate resources into growth, reproduction, floral traits, and rewards such as nectar and pollen to attract pollinators (Galen 1999). However, tradeoffs exist between attracting pollinators and defending against herbivory, pathogens, parasites, pre-dispersal seed predators, and pollen thieves (Irwin et al. 2004). Here, we examined the importance of pollen thieving muscid flies (Diptera: Muscidae) to the reproductive success of *Polemonium foliosissimum* A. Gray (Polemoniaceae) also known as leafy Polemonium or sticky Polemonium. *P. foliosissimum* is a perennial herb, displaying a cluster of purple campanulate flowers between late-June through early-August. *Polemonium foliosissimum* is also described as gynodioecious, or producing perfect hermaphroditic flowers consisting of both male and female reproductive parts (Fig. 3), as well as female flowers lacking functional pollen within the same population (Meehan 1889).

Accordingly, theory dictates that female flowers in order to achieve maximum fitness within a gynodioecious population should optimally produce twice as many ovules as hermaphrodites to adjust to chance fluctuations in larger pollen loads (Burd 1994). Even though female *P. foliosissimum* produce higher seed sets than hermaphrodites (Clarke and Brody 2016) these same females experience trade-offs between fitness and pollinator attractiveness as they also tend to attract less pollinators (Raguso 2004, Ashman et al. 2005). This may help explain the reduction to floral advertisements such as corolla size and nectar, pollen rewards offered by females to pollinators and thus adversely affecting pollinator choices between hermaphrodites and females (Galen 1999, Ashman et al. 2002). These attributable differences in floral advertisement and rewards between *P. foliosissimum* flowers (Brody 1997, Vega-Frutis et al. 2012) makes for a sublime environment to observe pollen thieving choices that can have potentially intrinsic ramifications upon the extent of pollen limitation.

Pollen thieving has been described as the collection or consumption of pollen by a predator that transfers minimal to no pollen due to mis-matches with flower morphology, such as the inability to contact a plant's stigma(s) (Inouye 1980, Hargreaves et al. 2009). This indicates that rewards are being stripped from plants, such as pollen without the intention of pollination from some visitors. The attribution of pollen thieves, such as observed muscid flies stealing protein rich pollen (Waser & Price 2016) may lead to pollen limitation as described as having inappropriate quality or quantity of pollen to fertilize ovules (Campbell & Halama 1993, Ashman et al. 2005). Thus, pollen as the mode of male reproductive transmission can consequentially reduce female fitness via seed or fruit set if found to be pollen limited (Shykoff et al. 2003, Hargreaves et al. 2010) while also decreasing its own siring ability. Within *P. foliosissimum* herbivory threats include pre-dispersal seed predators *Hylemya* (*Delia*) sp. (Diptera: Anthomyiidae), ungulate *Odocoileus hemionus*, and pollen thieving muscid flies (Diptera: Muscidae) that threaten to derail fitness from both sexes as exacerbated by pollen limitation (Zimmerman 1988).

The costly loss of reproductive sources in context to pollen limitation may effectually cause *P. foliosissimum* to over-compensate pollen production (loads) (Young & Young 1992) in order to attract more reproductive benefits such is the case with multiple individual pollinator visits, or fewer substantial pollinator visitation events as expressed within Bet-Hedging stratagem (Burd 1994). Other floral attractive responses such as oviposition choices in *Hylemya* sp. on the undersides of sepals in both female and hermaphroditic flowers (Clarke and Brody 2015) has been shown to destroy as much as 80% of complete seed sets (Zimmerman & Brody 1998). This may dictate that pollinator visitation choice can be effectively altering and adversely affecting sex-ratios in *P. foliosissimum* (Clarke and Brody 2015).

Within the scope of attracting pollinators, reduced pollen availability or pollen stolen from flowers may lead to selection pressure to change the amount of pollen produced thus shifting energy into other resources or floral traits such as flower size, shape, and color (Hargreaves et al. 2009). Pollen thieving can also effect neighboring floral communities that are dependent on the same pollinators (Johnson & Steiner 2000, Wirth et al. 2011, Waser & Price 2016) such that similar niche communal roles can be stressed and potentially intensified by

pollen limitation which may lead to more intra- and inter-specific competition (Hargreaves et al. 2009). The potential for whether pollen thieves have the capability to render hermaphroditic *P. foliosissimum* functionally female in respect to losing any potential advantages over female flowers will be investigated throughout this study. As well as this investigation will focus on the extent of pollen thieving muscid flies on pollen limitation within *Polemonium foliosissimum* populations and the effect of pollen thieving on hermaphrodite and female fitness as measured by seed set quantity and quality.

Methods

Measure of Pollen Limitation

Ten plots or neighborhoods of about 2 m^2 were created with three pairs of hermaphrodite and female focal plants matched by similar stage of maturity and size for a total of six focal plants per neighborhood (Fig. 1). Neighborhoods were separated by a minimum three-meter buffer zone between each other (as visualized by GIS using a Trimble 6000 Series GeoXT Explorer in Fig. 4). Before the emasculation manipulations occurred called stigma “pre-treatment” we collected 2-3 stigmas in total from abscised flowers on control stalks (stalks not hand-pollinated) from each focal plant. Four stalks of similar size and phenology were used for the control and hand-pollinated stalks. We then reduced the amount of pollen in the neighborhood by plucking half of the anthers and rudimentary anthers (2-3 per flower) from all neighboring hermaphroditic and female *P. foliosissimum* plants within two meters of any focal plant. The emasculation manipulation was split off into “evens” and “odds” days in which half of the plots would be experiencing the anther emasculations while the other plots were not emasculated. In order to treat for the potential effect of damage constituting pollinator visitation choice, the neighborhoods not experiencing emasculations (anthers being plucked by forceps) were instead experiencing the same handling and both anthers and rudimentary anthers were pinched instead. All the calyces from focal plant flowers used in this experiment were marked with varying colored paint pens in order to track the same sampled flowers’ seed sets at the end of the experiment in which colors match particular treatments. Five days after treatments were imposed, we collected stigmas from withered flowers on control stalks that had been open during the treatment, thus denoted as stigma “post-treatments.”

To examine if pollen reduction in the neighborhood of focal plants lessened pollen loads, we counted *Polemonium* pollen deposition on stigmas (Fig. 2) collected prior to anther removals and those collected after anther removals, emasculations. Stigmas were stained using basic fuchsin stain and examined under low (10x, 40x) magnification (Kearns and Inouye 1993). Emasculation manipulations were followed by two days of hand-pollination, in which bouquets of anthers were taken from pollen bagged *P. foliosissimum* outside the three-meter buffer zone around plots in the open meadow. Flowers used for the hand pollination were mixed and a minimum of two randomly selected flowers’ anthers were used to pollinate stalks marked for hand-pollination, as well as to introduce mate choice options with male siring (Hargreaves et al. 2008, Clarke and Brody 2015). Genetic variation was preserved by using anther bouquets from multiple plants and bouquets were only used for hand-pollinating a maximum of three focal plants before being renewed by a new set of anthers.

Stigmas were also collected from abscised “post-treatment” flowers that had experienced their pollen neighborhood being reduced by half on the second hand-pollination day. Following the hand-pollination days, a rest day was given to allow time for neighborhoods to rebound from manipulations. We also recorded total open flower counts and total flowering stalks for every focal plant the same day as post-stigmas were collected to track plant phenology. Both “odds” and “evens” plots were manipulated with emasculations twice, seed set is expected to be collected per focal plant and organized based off of treatments in the following weeks to determine overall fitness of the plants in the experiment as based off of seed set or fruit set weight and quantity.

Pollination visitation Rates

Pollinator visitation rates were collected in intervals of fifteen minuet observations from *P. foliosissimum* plants not used in the experiment. Visitation for pollinators, pollen thieves, and other was observed throughout the open meadow at various times of day to best represent the types of visitors and the frequency of pollinators to hermaphroditic and female flowers (Engel and Irwin 2003, Clarke and Brody 2015). As to the determination of sex-ratio in the meadow, all neighbor *Polemonium* plants in the plots had been identified and surveyed in order to track changes in sex-ratios and phenology between females and hermaphrodites in the future. Demographic information pertaining to the *Polemonium* in the meadow has also been initiated using four-meter transects.

Statistical analysis

Data was analyzed using a One-way ANOVA in JMP to apply the limited sample size of plants and to compare the nested plots within the research meadow. The same focal plants were being used throughout the study, but individual flowers account for unique independent data and are effected by the stigma pre-treatment, stigma post-treatment with respect to looking at fitness (seed set amount and weight), pollen deposition, and pollen visitation rate.

Results

Pollen Limitation

Although it was expected that emasculation would correlate to pollen limitation in stigma post-treatments as compared to pre-treatments (not emasculated but pinched neighborhood *Polemonium*), there was no significant difference between post and pre-treatments in regard to pollen limitation or anther removals ($p=0.3892$) see (Table 1. and Fig. 4). Rather the pollen load varied significantly between sexes, ($p<0.001$) in *Polemonium foliosissimum*. After taking data from both control and hand-pollinated stalks it was very clear that hand-pollinated stigmas have a higher pollen load and that counting these stigmas during treatments would not be provincial to look for pollen limitation.

Pollinator Visitation Rates

The average pollinators or visitors within the fifteen minute intervals were on average more representative of muscid fly visitation over *Bombus* sp. (Hymenoptera) (Fig.6). Granted there is

also the mis-representation of apparent visitors due to the limitation of pollinator visitation observations analyzed (not all data has been analyzed). There is a strong potential correlation with higher rates of muscid fly pollen thieves over bumblebees. As well as the numbers of flowers visited between pollinators did not substantiate to significant differences between the sexes ($p=0.0539$) see Fig. 7. However, pollinators and visitors are spending significantly more time at hermaphroditic flowers over female flowers ($p<0.001$) throughout the fifteen minuet interval observations (Fig. 8).

Discussion

Although there was no significant difference between pre-treatment pollen deposition and post-treatment pollen deposition this season, pollen limitation has been seen within the same population of *P. foliosissimum* in previous years (Brody unpub. data). The variation of pollen limitation may be attributable to lower levels of rain fall and snow pack that possibly affected the range and abundance of pollinators available this season. As well as phenological differences in *Polemonium foliosissimum* that may reflect less flower production this season as well. As such it has also been observed that pollen thief muscid fly populations intensified this season versus previous seasons and this could contribute to higher pollen consumption than average, thus radically reducing pollen from the populations in general. The pollen visitation rates from fifteen minuet interval observations have shown that hermaphrodites are visited more often and for longer extents of time most likely due to the availability of protein-rich pollen rewards that are not offered by female flowers. Although the number of flowers visited per plant were not significant between sexes, the average abundance of pollinators and pollen thieves significantly chose hermaphrodites over females. As such pollen thieves as shown to be the most abundant average visitor have the potential to effect hermaphrodite's functional male fitness capabilities in *Polemonium foliosissimum*. Pollen thieves as well could perpetuate additive effects in counteracting the cost of being female in a gynodioecious populations when pollen limitation is found. Due to the removal of advantageous pollen rewards from hermaphrodites, thus partially eliminating male siring capabilities, it may be expected that pollen limitation would affect future sex ratios within gynodioecious populations. However, this study has yet to correlate pollen limitation with fitness in *P. foliosissimum* due to lack of evidence of pollen limitation this season.

*Seed set will be collected in the up-coming weeks and analyzed to look at overall fitness between hermaphrodites and females via quantity and quality (weight) of fruit set. If females do produce more fruits than hermaphrodites this would help show that they are balancing out hermaphrodite advantages from producing pollen. However, since hermaphrodites losing pollen to pollen thieves are essentially functionally female, sex-ratio could be expected to shift slightly in favor toward females (but further analysis and future studies would be needed to support this claim).

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Tables and Figures

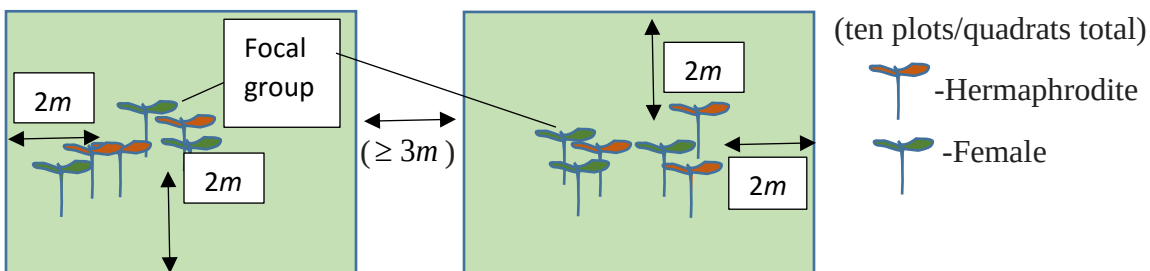


Figure 1. Representative plot neighborhood set-up.



Figure 2. *P. foliosissimum* pollen, picture taken by Irina Chen using a Leica 3000 Compound microscope camera extension.

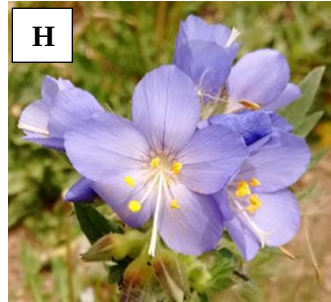


Figure 3. Representative female (F) and hermaphrodite (H) *P. foliosissimum* flowers. Photos taken by Brittany N. Smith

Table 1. Pollen Limitation data

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	13	72067.14	5543.63	6.8229
Error	514	417626.04	812.50	Prob > F
C. Total	527	489693.18		<.0001*

Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Pre/post	1	1	12073.381	14.8595	0.0001*
Site	9	9	15909.059	2.1756	0.0224*
Sex	1	1	44011.482	54.1678	<.0001*
A removal	1	1	34.582	0.0426	0.8366
Pre/post*A removal	1	1	603.333	0.7426	0.3892

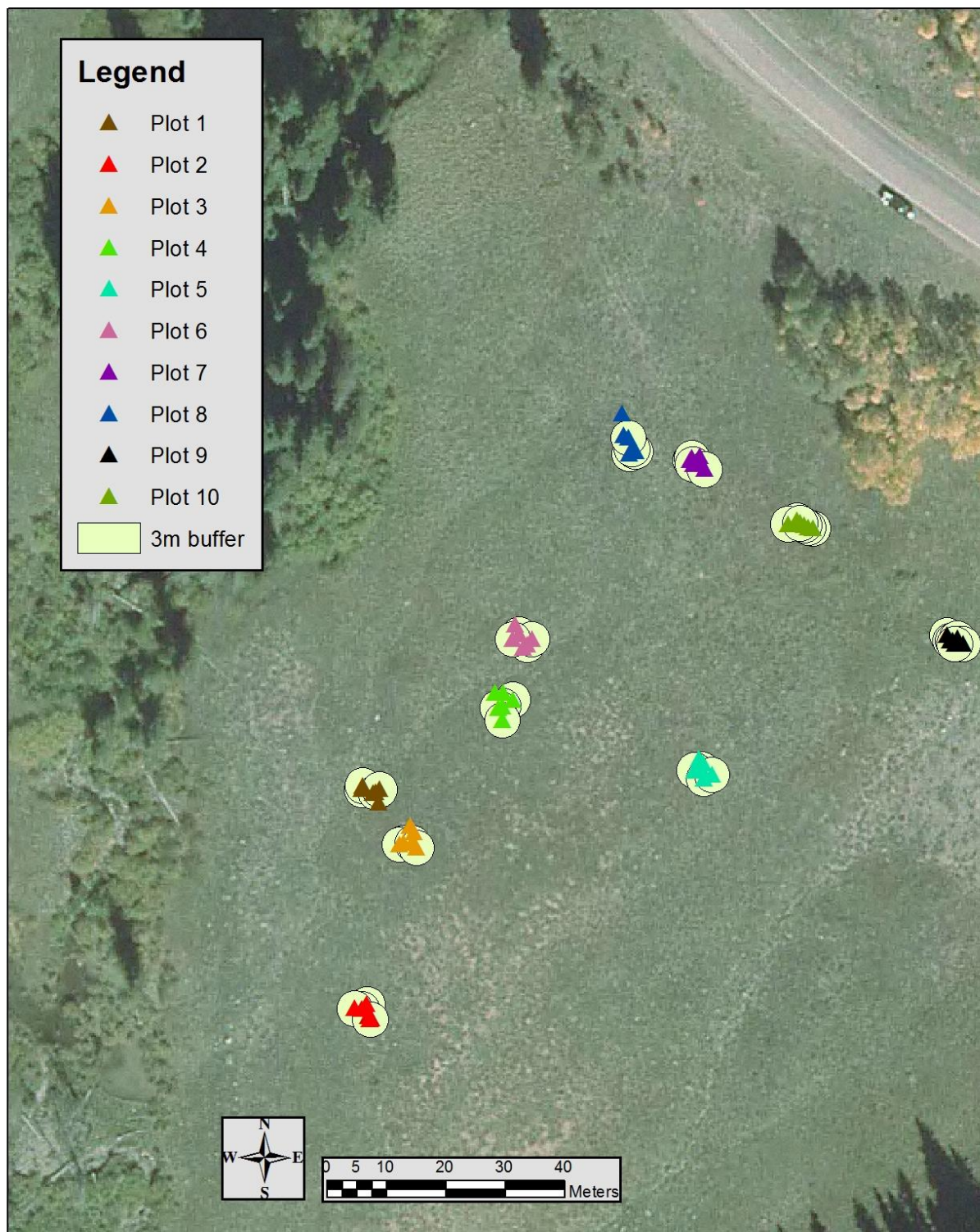


Figure 4. GIS map of Kebler Pass Road Site with the three-meter buffer zone around plots.

Average *P. foliosissimum* pollen deposition/stigma

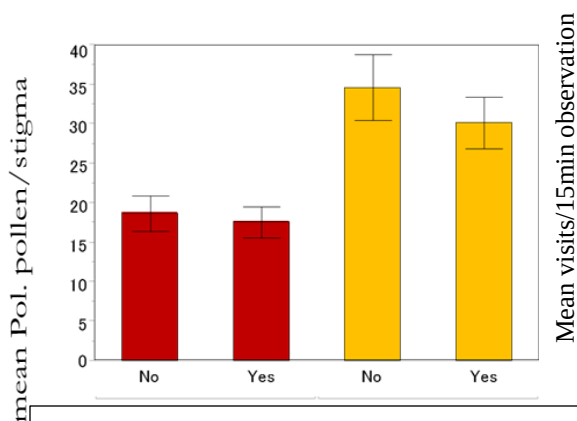


Figure 5. *Polemonium* pollen deposition in emasculated treatments (yes) and unemasculated treatments (no). H are hermaphrodites while F represents females.

Average Pollinator visits/ 15 min observation

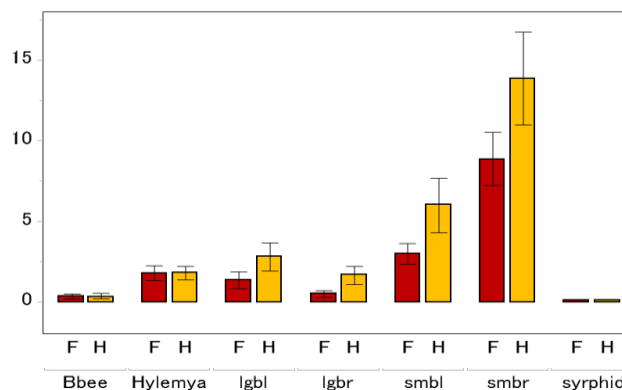


Figure 6. Smbl, smbr represent small black and small brown muscid flies, while lgbl and lgbr equal large black and brown muscid flies.

Average flowers visited/ observation

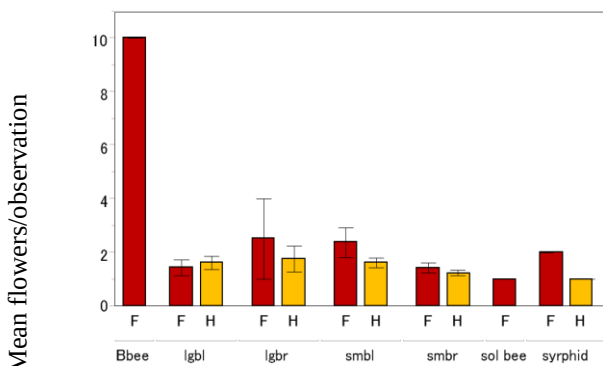


Figure 7. Smbl, smbr represent small black and small brown muscid flies, while lgbl and lgbr equal large black and brown muscid flies.

Average time(s) of visit/ flower

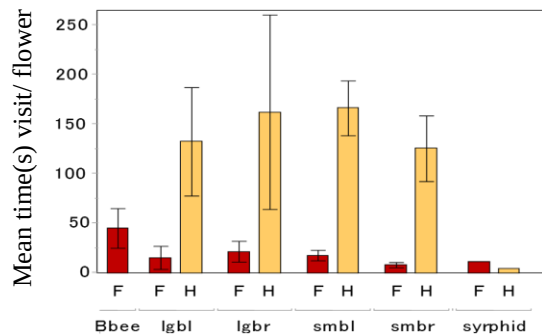


Figure 8. Time spent while visiting flowers is significantly higher on hermaphrodites than females ($p < 0.001$).