Frequency-dependent pollinator discrimination acts against female plants in the gynodioecious *Geranium maculatum*

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• **Background and Aims** Gynodioecy, the co-occurrence of female and hermaphroditic individuals, is thought to be an intermediate step between hermaphroditism and separate sexes, a major transition in flowering plants. Because retaining females in a population requires that they have increased seed fitness (to compensate for the lack of pollen fitness), factors that affect seed fitness are of great importance to the evolution of this mating system and have often been studied. However, factors negatively affecting female fitness are equally important and have been largely neglected. One such factor stems from female flowers being less attractive to insects than hermaphrodite flowers, thereby decreasing their relative fitness.

• **Methods** To test the severity and consequences of this type of pollinator discrimination in *Geranium maculatum*, experimental populations with the range of sex ratios observed in nature were created, ranging from 13 % to 42 % females. Pollinators were observed in order to measure the strength of discrimination, and pollen deposition and seed production of both sexes were measured to determine the fitness consequences of this discrimination. Additionally a comparison was made across the sex ratios to determine whether discrimination was frequency-dependent.

• **Key Results** It was found that female flowers, on average, were visited at half of the rate of hermaphrodite flowers, which decreased their pollen receipt and seed production. Additionally, females were most discriminated against when rare, due to both changes in the pollinators' behaviour and a shift in pollinator composition.

• **Conclusions** The results suggest that pollinator discrimination negatively affects females' relative fitness when they are rare. Thus, the initial spread of females in a population, the first step in the evolution of gynodioecy, may be made more difficult due to pollinator discrimination.

Key words: *Geranium maculatum*, Geraniaceae, gynodioecy, pollinator discrimination, minority disadvantage hypothesis, frequency-dependent fitness, plant mating system, plant–pollinator interaction.

INTRODUCTION

Plants exhibit a wide array of breeding systems, ranging from male and female functions housed within the same flower (hermaphroditism) to separating sexual functions on different individuals (dioecy; Richards, 1997). The shift from hermaphroditism to dioecy is a major evolutionary transition in flowering plants and most likely proceeds through gynodioecy, a system in which female individuals invade the ancestral hermaphroditic population (Charlesworth and Charlesworth, 1978; Webb, 1999; Weiblen *et al.*, 2000; Dufay *et al.*, 2014). The subsequent successful maintenance of females is determined largely by the seed fitness of females relative to that of hermaphrodites. Theoretical work has shown that, depending upon the details of the genetic control of sex, the seed fitness increase needed for females to be maintained ranges from just slightly higher to twice that of hermaphrodites (Lloyd, 1974; Charlesworth and Charlesworth, 1978; Charlesworth, 1981; Gouyon *et al.*, 1991). While many studies have focused on the cause(s) of enhanced female seed fitness (reviewed in Shykoff *et al.*, 2003; Dufay and Billard, 2012), of equal importance are the variety of processes that may decrease female fitness.

One such ecological process possibly decreasing female fitness is pollinator discrimination against female flowers. Discrimination between floral morphs has been documented in several gynodioecious species (reviewed in Delph, 1996) where female flowers were visited less often than sympatric hermaphroditic individuals. This behaviour pattern is likely due to female flowers being less apparent to pollinators (smaller or fewer flowers; Delph, 1996; Shykoff *et al.*, 2003) and often having fewer floral rewards than hermaphrodites (no pollen or less nectar; reviewed in Delph, 1996; Talavera *et al.*, 1996; Eckhart, 1999; Molano-Flores, 2002; Díaz and Cocucci, 2003). This pollinator discrimination against females could decrease relative female fitness if plants are pollen-limited, i.e. seed production increases with greater pollen deposition. Given that pollen limitation is common among gynodioecious species (Molina-Freaner and Jain, 1992; McCauley and Brock, 1998; Widen and Widen, 1999; Ashman and Diefenderfer, 2001) and other plant species in general (Ashman *et al.*, 2004; Knight *et al.*, 2005), pollinator discrimination is likely to influence the evolutionary dynamics of gynodioecious species.

Less well studied is the effect of changes in pollinator discrimination due to population context. In particular, the minority disadvantage hypothesis suggests that pollinators prefer the common morph in polymorphic populations, leading to a form of positive frequency-dependent fitness (Levin, 1972; Epperson and Clegg, 1987; Rausher *et al.*, 1993; Smithson and Macnair, 1996, 1997;

Smithson, 2001). For example, when two morphs of *Phlox drummondii* differing in floral shape were placed at different frequencies in an artificial population, pollinators visited the rare morph significantly less often than expected based on the relative abundances of the morphs, despite both morphs producing the same level of rewards (Levin, 1972). Similar results were found in the common morning glory, *Ipomoea purpurea*, with respect to corolla colour polymorphisms (Epperson and Clegg, 1987; Rausher *et al.*, 1993). This change in pollinator behaviour could lead to a positive correlation between frequency and reproductive output; when females are the rarer sex, such as during their initial invasion of a population, stronger pollinator discrimination against females could lead to reduced seed production. This added layer of frequency-dependent pollinator discrimination, if occurring, may make the establishment and maintenance of females difficult in the early evolution of gynodioecy.

Although several studies have found the predicted positive correlation between female frequency and seed production (Delph, 1990; Ashman, 1999; Delph and Carroll, 2001; Asikainen and Mutikainen, 2003; Vaughton and Ramsey, 2004; Nilsson and Ågren, 2006), these studies were mainly based on correlative data using unmanipulated population sex ratios and hence cannot speak to whether sex ratio or other confounding mechanisms might have led to the observed results. In order to isolate the effect of sex ratio on the flower–pollinator interaction, we experimentally manipulated the sex ratio using arrays of plants to investigate how pollinators interact with female and hermaphroditic flowers of the gynodioecious perennial *Geranium maculatum*. Specifically, we addressed the following questions: (1) whether pollinators discriminate against female flowers; if so (2) whether pollinator discrimination affects seed fitness of females; and (3) whether sex ratio in the population affects the degree of pollinator discrimination and thereby the relative fitness of the sexes. Because of their smaller petal size and lack of pollen, we expected that females would be discriminated against, especially when at lower frequencies, which would decrease females' relative fitness.

METHODS

Species description

Geranium maculatum is a gynodioecious, rhizomatous perennial ranging from south-eastern USA to Canada and west to the Great Plains (Radford *et al.*, 1968). Flowering begins in early spring (April to May in the study locations) and flowers are visited by generalist pollinators, including bees, flies and butterflies (M. L. Van Etten, pers. obs.). Flowers of both sexes contain ten ovules, producing at most five seeds. Females have small aborted anthers and smaller petals when compared with hermaphrodites (Ågren and Willson, 1991; Chang, 2006). Hermaphrodite flowers are protandrous, with two sets of five anthers dehiscing prior to the stigma lobes reflexing and becoming receptive, a process taking ~2–3 days under greenhouse conditions (Willson *et al.*, 1979; M. L. Van Etten, pers. obs.). Although the stigma remains receptive for 1–3 days (Willson *et al.*, 1979), once pollinated the stigma lobes close and the petals fall off soon after (M. L. Van Etten, pers. obs.). It generally takes less time following anthesis for the stigma lobes to extend and become receptive in female flowers than in hermaphrodite

flowers (S.-M. Chang, pers. obs.), but it is not clear whether the two sexes differ in how long the stigma remains receptive. Hermaphrodites are self-compatible though selfing rates are low (0–5 %) in natural populations (Van Etten *et al.*, 2014). Seeds mature ~1 month after pollination and are dispersed by the elastic dehiscence of the schizocarp. Although flower number per inflorescence is approximately the same between sexes in natural populations (Ågren and Willson, 1991; Chang, 2006), females produce more seeds than hermaphrodites (Ågren and Willson, 1991; Chang, 2006) and seeds that have a higher germination rate in the greenhouse (Chang, 2006). Based on field and greenhouse observations, sex is genetically determined. Preliminary data rule out the possibility that sex is controlled entirely by cytoplasmic genes, but whether cytoplasmic and nuclear genes are both involved has not yet been determined (M. L. Van Etten and S.-M. Chang, unpubl. data). Local populations around Athens, GA, range from 0 to 50 % female (Chang, 2006).

Array experiment design

Seeds were collected from female (F) and hermaphroditic (H) plants in two populations in Athens, GA, in 2003 and were germinated and grown in the greenhouse. They were later placed outdoors in a pollinator exclusion enclosure for 2 years prior to this study. In the spring of 2008, we randomly selected plants with several buds to create arrays with sex ratios of 13 % (4 F, 27 H), 26 % (8 F, 23 H) and 42 % females (13 F, 18 H), hereafter referred to as low, intermediate and high female frequency, respectively. These ratios were selected to span the range of sex ratios observed in local populations. Three locations similar in background vegetation type to *G. maculatum*'s natural populations but isolated by at least 50 m from any *G. maculatum* were chosen within the Georgia State Botanical Gardens (Athens, GA), each housing one sex ratio. In each location, a grid was created such that each plant would be 0·5 m from its nearest neighbours, with six or seven plants per row, giving a total of 31 plants per location. Positions of the study plants within a location were randomly assigned. After at least six 2-h pollinator observations were completed in every location (six for each treatment in the first two rotations and ten for the third), the sex ratios were rotated among locations, leading to a Latin square design, so that for each time period a sex ratio was represented in one of the three locations and by the end of the experiment each location had housed each of the sex ratios once for a consecutive period of 3–8 d, during which six to ten observations were completed. This design led to each treatment at each location having between 11·5 and 20·5 h of observation, with a total of 131·4 h of observation over the entire experiment. For each rotation, plants were randomly reassigned to sex ratio treatments and locations within treatments.

Data collection

Pollinator observations were carried out on warm (~21–27 °C), sunny days in two time periods: 1000–1230 and 1500–1700h. For each pollinator observation period, each of the three locations was observed by a single observer simultaneously for 2 h, with observers rotated among locations. For each pollinator observed, we recorded its type (see more details below), the plants and the

number of flowers it visited and the amount of time it spent on each flower. Pollinators were followed until they left an array. In the rare event that more than one pollinator entered the array simultaneously, we followed the first pollinator until it left the array before following the next pollinator. Because of this, our observation represents an underestimate of the actual visitation. Pollinators were broadly grouped into size classes because we predicted that size was likely to determine an insect's effectiveness in pollinating *G. maculatum* flowers. Bees $\sim$0·75 cm long were categorized as small bees, most likely small sweat bees (Halictidae), and were thought to be ineffective pollinators because they are too small to consistently contact the stigma lobes. Bees between 0·75 and 1·3 cm long were categorized as medium bees and consisted of honey bees (*Apis mellifera*) and green and black sweat bees (Halictidae). Flies (primarily syrphid flies, Syrphidae) were generally of the same size as the medium bees but were grouped separately because they generally behaved differently from bees. Large bees were >1·3 cm and included primarily carpenter bees (*Xylocopa*), but also bumblebees (*Bombus*) and other large unidentified species. The medium and large bees and flies were considered effective pollinators because they are large enough to come into contact with the stigma lobes while collecting nectar or pollen. Other pollinators, such as butterflies, wasps and beetles, were observed infrequently ($\sim$5 % of the total visits) and were therefore excluded from our analysis.

For each observation time, we also recorded the number of open flowers on each plant. Because individual plants could be used in different sex ratio treatments during different parts of this study, to identify the sex ratio treatment that a particular flower received we labelled the lower surface of the sepal for each flower with a receptive stigma with a small dot of DecoColor paint (Marvy Uchida, CA, USA), using a different colour for each day and location combination. If a flower was receptive in two different sex ratio treatments then its data were discarded (16 out of 931). The use of DecoColor had been tested in our pilot studies and showed no effects on the seed maturation in this species. To determine pollen deposition, stigmas, collected after fruits began to develop, were placed under a dissecting microscope to count the number of pollen grains on the stigmatic surface ($n_F = 374$, $n_H = 529$). Fruits were collected when mature and seeds were counted.

We also measured a variety of other plant traits for each of the plants used in the array. We measured the areas of two fully expanded petals per plant from different flowers using ImageJ (Abràmoff *et al.*, 2004). For hermaphrodites, pollen grains on two anthers were collected using fuchsin jelly (Kearns and Inouye, 1993) and mounted on a glass slide. These slides were photographed using a digital camera (Leica DC200, Leica Microsystems, Wetzlar, Germany) mounted over a dissecting microscope and the photographs were analysed using ImageJ to obtain the total number of pollen grains. To obtain a measure of plant size, rhizomes were weighed after the completion of the experiment to the nearest milligram after removing the soil.

In a separate experiment, to determine whether nectar production differs between sexes, we collected nectar from greenhouse plants of similar age and progression into flowering. Flowers from females and hermaphrodites were collected when the stigma lobes were open. Because of the very small amount of nectar in each flower, flowers were centrifuged to remove nectar (Heinrich, 1983), which was then collected using a 5-µL micropipette with fine tips (Drummond, PA, USA) and measured to the nearest mm using a dissecting microscope ($n_F = 13$, $n_H = 11$).

Data analysis

Morphological differences between the sexes (average flower number open per day and petal size) were tested using an ANCOVA in SAS v9.3 (SAS Institute, 2012) with sex as the main effect and final rhizome size as a covariate to account for plant size differences not intended for this study. Nectar amount and final rhizome size were analysed using a *t*-test to determine whether sexes differed.

For visitation and reproduction variables, we generally used summary data calculated as the mean of the data recorded for each plant in a particular sex ratio and location combination. Because plants often produce different number of flowers on the day of observation, we standardized these measurements before the analyses with the number of flowers open on that day to evaluate how the main predicting variables performed if all plants produced the same number of flowers. We chose to standardize measures in this way because, while in this experiment females produced fewer flowers than hermaphrodites per observation time, multiple previous studies have shown that *G. maculatum* females generally produce an equal number of or more flowers (Ågren and Willson, 1991; Chang, 2006; Van Etten *et al.*, 2008). Results on a per plant basis were similar to those on a per flower basis and can be found in the Appendix. Due to females producing fewer flowers, the flower sex ratio was lower than the plant sex ratio. However, this does not affect our results because our measure of discrimination (the difference between visitation rates between females and hermaphrodites) is independent of the sex ratio.

For the visitation data, we calculated two variables from the field records for each plant at a given sex ratio: mean visitation rate and mean duration on a flower. First, the mean visitation rate was calculated as the total number of flower visits to a particular plant per observation hour divided by the number of flowers on the plant at that time (i.e. mean visits per flower h^{-1}). This was also calculated separately for the different types of pollinators. Second, we calculated the mean duration that a pollinator stayed on a flower (seconds per flower). Each plant in a given array (sex ratio × location) combination is represented by one value for each variable listed here, giving a total of 31 observations for each array. Two models were used to analyse the above measures. First, each visitation measure was analysed using an ANOVA in SAS (SAS Institute, 2012) with week, location, sex ratio, sex and sex × sex ratio as predictors. Second, to examine whether flower size affected visitation we included petal size as a covariate in the visitation rate analysis. These analyses assume that total flower number does not affect the visitation rate per flower. To confirm that this did not change our results, we included open flower number as a covariate in the above analyses. Flower number was only significant for visitation rate for small bees ($P = 0·01$) and the interpretation of the results from the simpler model did not change. Therefore, we have not included these analyses in the results.

For the post-pollination measures we calculated mean pollen deposition on the stigma and seed production per flower. Similar to the visitation measures, we calculated one value per

individual per sex ratio × location combination. Average pollen deposition per flower was modelled using week, location, sex ratio, sex and sex × sex ratio as predictors. To understand how visitation rates affected pollen deposition we included the visitation rate and duration as covariates in the above model. Least-squares means (LSMs) from this model were used to estimate the pollen deposition expected after accounting for visitation differences, i.e. when visitation rates were held equal between sexes. Because many plants did not produce any fruits in a given sex ratio treatment (∼47 % of plants produced no fruit in a treatment), we analysed seed production per flower using a zero-inflated negative binomial model (Ridout *et al.*, 1998). This model assumes that zeroes in our data were likely to be produced due to two different processes leading to an excess of zero values compared with a normal negative binomial model. The first process is responsible for the probability that a plant may produce any fruits at all and is modelled using a logit model. This model is also referred to as the 'zero model' of the analysis because most of the zeroes, though not all, in our data will be explained by this model. The second process is responsible for the number of seeds that a plant would produce and is modelled using a negative binomial model. For the zero model we chose to include sex, final rhizome weight and their interaction as predictors under the assumptions that the reason for the lack of seeds was due to resource availability (represented by rhizome weight) and that this could act in a sex-specific manner. For the negative binomial model we included week, location, sex ratio, sex, sex × sex ratio and rhizome weight as predictors. To understand how visitation rates affected seed production, we performed an additional analysis that also included visitation rate and duration as covariates in the negative binomial model. The LSMs from this model were used to estimate the seed production expected after accounting for visitation differences. Additionally, to understand how pollen deposition affected seed production we performed an analysis that included pollen deposition as a covariate in the negative binomial model. These analyses were performed using PROC GENMOD in SAS (SAS Institute, 2012).

To satisfy the normality assumptions of the analyses, the following variables were transformed: duration was log transformed; pollen deposition was square root transformed; and Box–Cox transformation was used for visitation rate ($\lambda = 0.25$).

RESULTS

Morphological differences

Females in this study produced about half as many flowers ($F_{mean} = 1.81 \pm$ s.e. 0.23 flowers per observation period, $H_{mean} = 2.86 \pm 0.14$; $F_{1,119} = 15.45$, $P < 0.0001$) and ∼40 % smaller petals than hermaphrodites ($F_{mean} = 3855.73 \pm 288.74$ mm^2, $H_{mean} = 6733.26 \pm 172.70$ mm^2; $F_{1,114} = 72.69$, $P < 0.0001$), despite similar final rhizome sizes ($F_{mean} = 115.0 \pm 10.05$ g, $H_{mean} = 126.5 \pm 6.05$ g; $t_{122} = 0.97$, $P = 0.33$). Females and hermaphrodites produced similar amounts of nectar per flower ($F_{mean} = 1.17 \pm 0.11$ µL, $H_{mean} = 1.18 \pm 0.12$; sex, $F_{1,22} = 0.00$, $P = 0.96$). Hermaphrodites on average produced 512 ± 9.25 ($n = 80$) pollen grains per anther, giving an estimated 5120 pollen grains per flower.
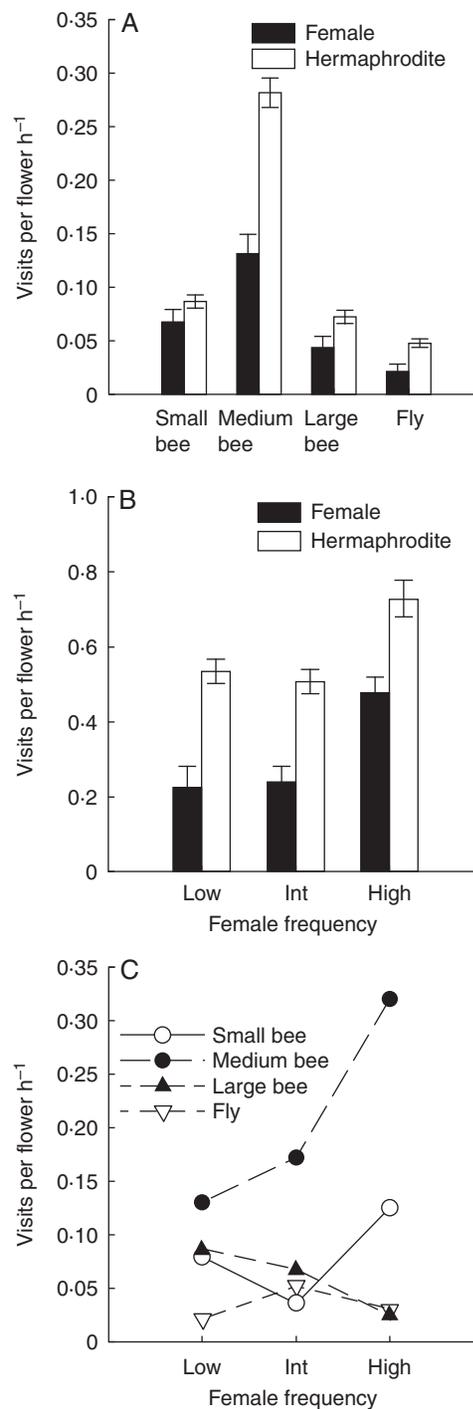

FIG. 1. (A) Visitation rates (LSMs ± s.e.) for females and hermaphrodites (as indicated in key) for each pollinator class. (B) Average total visitation rate for females and hermaphrodites for each sex ratio. (C) Visitation rates by pollinator class for each sex ratio treatment.

Pollinator discrimination

All but the small pollinator types discriminated against females (Fig. 1A), resulting in female flowers having about half the visitation rate of hermaphrodites ($F_{lsmean} = 0.30 \pm 0.03$ visits per flower h^{-1}, $H_{lsmean} = 0.58 \pm 0.02$; $P < 0.0001$;

TABLE 1. *Results for visitation measures including* F *values for each predicting variable. Numbers in parentheses in column headings are d.f.*

	Sex (1)	Sex ratio (2)	Sex × sex ratio (2)	Location (2)	Week (2)	Error d.f.
Visitation rate (visits per flower/h^{-1})	51·14***	15·57***	1·11	58·52***	63·10***	286
Duration (s per flower per visitor)	2·73	2·38	1·64	11·55***	14·83***	274
Small bee visitation rate	2·09	20·15***	0·01	80·38***	13·48***	286
Medium bee visitation rate	38·52***	22·94 ***	0·24	26·63***	83·91***	295
Large bee visitation rate	5·95*	11·03***	0·84	114·57***	27·47***	286
Fly visitation rate	10·44**	4·82**	1·36	14·68***	27·67***	286

*$P < 0.05$, **$P < 0.01$, *** $P < 0.0001$.

Table 1). Medium bees were the most frequent pollinator type for both females and hermaphrodites, followed by small bees and large bees (Fig. 1A). Petal size was a significant predictor for visitation rates ($F_{1,277} = 18.21$; $P < 0.0001$), with plants with larger petals receiving more visits. However, sex remained significant ($F_{1,277} = 11.41$; $P = 0.0008$) even when petal size was included in the model, suggesting that sex differences besides petal size also influenced visitation rates. In contrast to visitation rate, pollinators spent similar amounts of time per flower on both sexes ($F_{lsmean} = 8.75 \pm 0.83$ s, $H_{lsmean} = 10.40 \pm 0.46$; $P = 0.16$; Table 1).

The interaction between sex and sex ratio was not significant for any measure of visitation (Table 1). However, there was a trend for stronger discrimination when females were rarest; at low frequency females had a 58 % lower visitation rate than hermaphrodites, at intermediate frequency a 53 % lower rate and at high frequency a 35 % lower rate (Fig. 1B). This trend of increased discrimination when females were rare was fairly consistent across pollinators (small bees, low 24 %, intermediate 30 %, high 17 %; medium bees, low 67 %, intermediate 56 %, high 41 %; large bees, low 49 %, intermediate 32 %, high 32 %; flies, low 66 %, intermediate 60 %, high 35 %), providing some support for the minority disadvantage hypothesis.

The relative frequency of pollinator types changed with array sex ratios, primarily due to an increase in visitation rates of medium pollinators as female frequency increased (Fig. 1C, Table 1). This led to a shift in the pollinator composition, with medium bees making up an increasing proportion and large bees a decreasing proportion as female frequency increased.

Both week and location affected the visitation measures (Table 1). Overall, pollinator visitation rate decreased over time ($P < 0.0001$), except for the small bees, for which the rate increased over time ($P < 0.0001$). Visit length per flower increased over time ($P < 0.0001$). The ranking of locations in their visitation rates differed among pollinator types. However, one of the locations (A3) had the lowest visitation rate for all but the medium bees ($P < 0.0001$). Visit duration also differed among locations ($P < 0.0001$), with A1 having the highest and A2 having the lowest duration.

Effect of discrimination

Flowers in the array received on average 45.3 ± 2.4 pollen grains during this study. Females on average had 26 % fewer pollen grains deposited than hermaphrodites ($P = 0.04$; Table 2, Fig. 2A). When including visitation rate as one of the predicting variables in the model, the sexes did not differ in pollen deposition ($P = 0.47$; Table 2, Fig. 2A), suggesting that differences in visitation rates account for the sex differences in pollen deposition. Including visitation rate in the model led to a better-fitting model (Table 2), further supporting the importance of visitation rate on pollen deposition. Sex ratio did not significantly affect pollen deposition ($P = 0.77$; Table 2). Pollen deposition decreased over time ($P < 0.0001$).

Increased visitation rates and pollen deposition significantly increased seed production ($P = 0.02$ and $P = 0.001$, respectively; Table 2). Including visitation rates or pollen deposition increased the fit of the model (Table 2), suggesting that plants may be visitor- and/or pollen-limited. Despite lower visitation rates and lower pollen deposition for females over all sex ratios, the two sexes did not differ significantly in seed production per flower regardless of whether visitation rate was included as a predicting variable in the model (visitation rate included, $P = 0.39$; not included, $P = 0.40$; Table 2, Fig. 2B). Like visitation rates and pollen deposition, seed production decreased over time ($P < 0.0001$).

Sex ratio did not significantly affect seed production ($P = 0.07$; Table 2, Fig. 2C). However, when looking across the three array types, females tended to produce fewer seeds in the low female frequency arrays than the other arrays (contrast $\chi^2_1 = 3.66$, $P = 0.056$) but not when visitation rate was accounted for in the model (contrast $\chi^2_1 = 1.28$, $P = 0.26$; Fig. 2C). This again suggests that the lower pollinator visitation rate at the lowest female frequency can account for the slightly reduced seed production in females when compared with females at higher female frequencies. This trend led to females producing 0·55-, 1·02- and 1·10-fold the number of seeds compared with hermaphrodites in the low, intermediate and high female frequencies, respectively.

DISCUSSION

Overall pollinator discrimination and its effects

Though pollinator discrimination has been demonstrated in several previous studies, our results emphasize how this phenomenon is influenced by the population sex ratio in *G. maculatum*. Through experimental manipulation of the sex ratio, we found that, on average, females received about half the number of pollinator visits per flower as hermaphrodites. While visitation rates for small bees were similar between the sexes, medium and large bees and flies strongly preferred hermaphrodites. This discrimination against females could be due to a variety of traits that pollinators tend to prefer, such as larger flowers (Bell, 1985; Delph

TABLE 2. *Results for reproductive measures, including* F *values for pollen deposition or* χ^2 *values for seed production. Numbers in parentheses in column headings are d.f.*

	Sex (1)	Sex ratio (2)	Sex × sex ratio (2)	Location (2)	Week (2)	Plant size (1)	Plant size × sex (1)	Visits per flower h^{-1} (1)	Ave flower duration (1)	Pollen deposition (1)	Error d.f.	AIC[1]
Pollen deposition	4·47*	0·27	0·04	1·85	15·61***						225	1127·7
Pollen deposition with visitation covariates	0·54	2·08	0·65	1·96	4·81**			10·51**	3·18		211	1042·9
Seeds per flower											n = 279	1361·3
Zero model	0·46					2·49	2·48					
Negative binomial model	0·74	5·34	1·77	4·64	29·49***	0·11						
Seeds per flower with visitation covariates											n = 266	1335·9
Zero model	0·00					1·11	1·06					
Negative binomial model	0·73	3·04	1·13	1·77	7·95	0·01		10·52**	0·22			
Seeds per flower with pollen deposition covariate											n = 226	1207·0
Zero model	0·78					2·40	1·95					
Negative binomial model	0·28	4·33	2·30	6·86*	23·47***	0·03				5·51*		

[1]Akaike's Information Criterion.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$.

FIG. 2. (A) Pollen deposition (LSMs ± s.e.) and (B) seeds per flower for females and hermaphrodites. (C) Seeds per flower for each sex for each sex ratio. Bars in (A), (B) and (C) are from a model where the effect of different visitation rates was not removed. Diamonds represent the predicted values when visitation rate differences were accounted for in the model, i.e. the predicted values when holding visitation rates the same between sexes. For more details see text.

and Lively, 1992), larger floral display (Schmid-Hempel and Speiser, 1988; Klinkhamer and de Jong, 1990; Eckhart, 1991; Mitchell, 1994; Galloway et al., 2002) and flowers containing pollen (Golding et al., 1999; Duffy and Johnson, 2011; Duffy

et al., 2013; Dötterl *et al.*, 2014), all of which hermaphrodites had in our study system. Pollinators could also be responding to more subtle cues that were not measured in this study, such as the scent of the pollen, as was found in *Fragaria virginiana* (Ashman *et al.*, 2005). In *G. maculatum*, there are glandular trichomes on the outer surface of the sepals which emit scents, but data are not available on whether females and hermaphrodites differ in this trait (S.-M. Chang, pers. obs.). Further manipulative studies would help to determine which traits are most important in pollinator preference in this species.

Regardless of the cause, we found that pollinators' discrimination against females decreased their pollen receipt and likely their seed production as well. Female flowers received 26 % less pollen than hermaphrodites, which is likely a direct consequence of lower visitation rates, given that females rely on pollinators to deposit pollen grains. Less clear-cut is the effect of visitation rates on seed production. Although visitation rates and pollen deposition were lower for females, the sexes did not differ significantly in seed production. This could be because females, on average, received sufficient pollen for the maximum number of seeds possible given the available resources, indirectly suggesting a lack of pollen limitation in this study. However, several lines of evidence suggest that pollinator discrimination could still play a role in seed production. First, visitation rate and pollen deposition showed significant effects on seed production, suggesting that pollen limitation occurred in this study, albeit at only the lower visitation rates. Second, when we accounted for differences in visitation rate, our analysis showed that females would have produced 20 % more seeds than hermaphrodites, given the same visitation rate. This provides some support for the possibility that pollinator discrimination when pollinators are infrequent could have a negative effect on the relative seed production of females – a parameter critical for the establishment of females in the gynodioecy system.

Frequency-dependent discrimination and its effects

One intriguing result we found was that pollinator discrimination against females was strongest when they were at the lowest frequency; females had a 58 % lower visitation rate than hermaphrodites at low frequencies versus 35 % lower rates at high female frequency arrays. Two frequency-dependent mechanisms have contributed to this pattern. First, as suggested by the minority disadvantage hypothesis (Levin, 1972), each type of pollinator showed a slight decrease in discrimination as females became more common. Second, the relative proportions of different pollinator classes shifted with female frequency. This shift in pollinator composition was not simply a temporal fluctuation of the insect community surrounding our study sites because every sex ratio was represented in any given temporal period during our study. Instead, the shift in pollinator composition appeared to be associated with the specific female frequency in the array, regardless of the times and the locations of the arrays (data not shown). These results suggest a genuine shift in pollinator composition in response to the population sex ratio; namely, a decrease in visitation rates by large bees and an increase by medium bees from low to high female frequency. A shift in pollinator assemblage was also found in *F. virginiana*, in which arrays with more females had relatively

fewer visits from pollen collecting flies (Ashman and Diefenderfer, 2001). Exactly how such shifts in the relative abundance of pollinators may translate into seed production cannot be determined without a measurement of the effectiveness of the pollinators on each sex and more comprehensive data on behaviour (e.g. constancy, nectar robbing rates and number of flowers or plants visited). Nevertheless, our results showed that frequency-dependent discrimination could occur through changes in individual pollinator behaviour (minority disadvantage hypothesis) and/or changes in the composition of the pollinator pool.

Context-dependent pollinator discrimination has only been examined in a handful of experimental and observational studies, which show contrasting patterns. Similar to our results, Case and Ashman (2009) found that, when at low frequencies, female plants in wild strawberries (*F. virginiana*) received significantly fewer visits than expected by chance, while at higher frequencies there was less of a difference. Ashman and Diefenderfer (2001) also found that in arrays with high female frequencies pollinators discriminated less against females. In contrast, McCauley and Brock (1998) compared relative fitness of females in manipulated arrays of *Silene vulgaris* and found the highest female fitness at the lowest female frequencies, but no pollination data were available to determine whether pollinator behaviour played any role in this contrasting result. Finally, another study using *Kallstroemia grandiflora* found no effect of female frequency on the level of discrimination (Cuevas *et al.*, 2008). Like the experimental studies, survey studies using natural populations that varied in their sex ratio also found contrasting results. In a study using three natural populations of an European congeneric species, *Geranium sylvaticum*, female plants were found to have significantly lower visitation rates than expected only in the populations with higher female frequency (Asikainen and Mutikainen, 2005). Alonso (2005), on the other hand, found that female *Daphne laureola* plants received higher pollen loads in populations with higher female frequencies than those with lower female frequencies. Unfortunately, population sex ratio was confounded with altitude in this study so their effects could not be decoupled. These studies together highlight two points for future consideration. First, there are not enough studies to allow conclusions on whether this context-dependent pollinator discrimination is common among gynodioecious species. In particular, we need more studies that experimentally manipulate sex ratio in order to determine its effect on pollinator behaviour and the resulting consequences for the evolution of gynodioecy. Second, judging from the contrasting patterns from different systems and studies, pollinator discrimination is likely to be an idiosyncratic process that is species- and/or context-dependent. However, as we have shown in this study, this process can play an important role in the process of trait evolution, particularly for floral traits, in natural populations and should be considered more often in future studies.

Implications for the evolution of gynodioecy in G. maculatum

The ultimate goal of this study was to understand how interaction between the gynodioecious *G. maculatum* and its pollinators may contribute to the establishment of females in natural populations. As mentioned in the Introduction, the successful

establishment of females requires seed fitness ranging from slightly higher (in the case of cytonuclear control systems) to twice as high as that of hermaphrodites (in the case of strictly nuclear control). Our results revealed a rather difficult situation for females to become established because their estimated relative fitness was lowest at low frequencies – only ~55 % of hermaphrodites' fitness. This low level of relative fitness in females will prevent them from establishing in a population regardless of the type of genetic control, unless other mechanisms, such as resource reallocation (Eckhart, 1992; Ashman, 1994), inbreeding avoidance (Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987; Schultz, 1999; Glaettli and Goudet, 2006) and/or environmental heterogeneity (Delph, 1990, 2003; Van Etten and Chang, 2009), also operate in natural populations to boost females' seed production.

Our results suggest that frequency-dependent selection on females can be more complicated than previously thought. In the gynodioecy literature, negative frequency-dependent selection manifested through pollen availability has often been invoked as a mechanism that can maintain both females and hermaphrodites in the same population (McCauley and Brock, 1998; Olson *et al.*, 2006; Zhang *et al.*, 2008). In that scenario, females are generally free from pollen limitation when rare because the abundant hermaphrodites provide sufficient pollen for the female's reproduction. In contrast, our results showed that females could still encounter pollen limitation when at low frequency due to the higher discrimination they encounter from pollinators, due to changes in either pollinator behaviour or pollinator assemblage. This positive frequency-dependent selection could in part explain results from several previous empirical studies that found a positive relationship between relative female fitness and female frequency (Delph, 1990; Wolfe and Shmida, 1997; Ashman, 1999; Delph and Carroll, 2001; Asikainen and Mutikainen, 2003; Ramula and Mutikainen, 2003; Vaughton and Ramsey, 2004; Nilsson and Ågren, 2006). However, most of these studies were examining field populations and therefore could not isolate the effect of sex ratio from other potential mechanisms that may account for the correlation. Combined, these results suggest that frequency-dependent fitness in gynodioecious systems may consist of two distinct processes: at low female frequencies, pollen limitation in females occurs due to high pollinator discrimination and at high frequencies it occurs due to too few pollen donors.

In this study we examined the importance of pollinator discrimination on the relative seed fitnesses of females and hermaphrodites, which is a major factor influencing the evolution and maintenance of gynodioecy. We found that most types of pollinators visited hermaphrodites more frequently than females and that this difference decreased pollen deposition. Additionally, discrimination tended to be stronger when females were rare. Considering these results in the context of the evolution of gynodioecy in *G. maculatum*, the establishment of females in populations should be rather difficult due to the discrimination they suffer from pollinators. This could potentially explain why female frequency is usually low in natural populations of *G. maculatum*, often between 3 and 35 %, and never exceeds 50 %. It is possible that, even when established, females might only be able to increase their frequency in the population under very favourable selection environments that occur infrequently. These conclusions are admittedly speculative

given our current knowledge of this gynodioecious species. Nonetheless, our results show that pollinators can play an influential role in determining the relative seed fitnesses of the sexes and thereby the maintenance of gynodioecy in *G. maculatum*.

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APPENDIX

Per plant ANOVA results. Numbers in parentheses in column headings are d.f.

	Female mean	Hermaphrodite mean	Sex (1)	Sex ratio (2)	Sex × sex ratio (2)	Location (2)	Week (2)	Plant size (1)	Visits per plant h^{-1} (1)	Error d.f.
Visits per plant h^{-1a}	0·40 ± 0·05	1·35 ± 0·08	74·89***	7·21**	2·63	21·57***	28·43***			288
Flowers per visit[b]	1·28 ± 0·06	1·43 ± 0·03	5·90*	1·32	0·61	0·48	1·83			263
Proportion of flowers visited per visitor[c]	0·63 ± 0·04	0·50 ± 0·02	8·97**	2·54	4·15*	1·23	4·17*			263
Visit duration per plant (s)[c]	12·41 ± 1·49	15·98 ± 0·80	4·10*	0·62	1·08	13·62***	17·06***			263
Seed number d^{-1d}	0·36 ± 0·07	0·35 ± 0·04	0·00	3·00*	1·50	1·75	12·04**	5·21*		282
Seed number d^{-1d} with visitation	0·66 ± 0·09	0·28 ± 0·03	18·29***	1·72	0·38	2·13	2·62	0·11	88·39***	281

[a]Log transformed.
[b]Box–Cox transformation, $\lambda = 1\cdot5$.
[c]Box–Cox transformation, $\lambda = 0\cdot5$.
[d]Square root transformed.
*$P < 0\cdot05$, **$P < 0\cdot01$, ***$P < 0\cdot0001$.