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RESEARCH ARTICL

Influence of local density and sex ratio on pollination in an ambophilous flowering plant

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PREMISE: Variation in local density and sex ratio in dioecious plants can affect mating success through the actions of pollen vectors, principally generalist insects or wind. Increased density and male-biased sex ratios should promote pollen transfer and seed production, but their combined effects have not been investigated for ambophilous species, which exhibit both insect and wind pollination.

METHODS: We manipulated density (low vs. high) and sex ratio (1:1 vs. 3:1 male-biased) in arrays of dioecious ambophilous *Thalictrum pubescens*. We quantified visitation rates and foraging times to examine whether pollinators exhibited sex-specific preferences and determined the seed set of arrays.

RESULTS: Pollinators visited more plants per foraging bout at high than low density. Visitation rates and foraging times of visitors were greater for male than for female plants but did not depend on the density or sex ratio of arrays. However, whereas solitary bees displayed a strong preference for males, hover flies were indifferent to plant sex phenotype. Solitary bees also visited significantly more plants per foraging bout than hover flies. There was a significant interaction between density and sex ratio on seed set. At low density, seed set was greater for 3:1 than for 1:1 arrays, but at high density the opposite pattern occurred.

CONCLUSIONS: The demographic factors we investigated had complex influences on pollinator foraging behavior and patterns of seed set. Several factors may explain our results, including the influence of density and sex ratio on pollen export from arrays, grooming by pollinators, and the contribution of wind pollination.

KEY WORDS ambophily; biased sex ratios; density; dioecy; insect pollination; Ranunculaceae; *Thalictrum*; wind pollination.

For immobile organisms such as plants, mating tends to occur predominantly between near neighbors and is therefore often density dependent (Levin and Kerster, 1974; Antonovics and Levin, 1980; Campbell, 1991; Smouse et al., 2001; Ghazoul, 2005; Robledo-Arnuncio and Austerlitz, 2006). The importance of density in determining the reproductive success of individuals may differ for plant species that are sexually monomorphic vs. polymorphic (Barrett and Thomson, 1982; Stehlik et al., 2006; Vamosi et al., 2006; Fromhage and Kokko, 2010). For example, hermaphroditic populations should be less sensitive to variation in density than dioecious species because most individuals encountered can serve as potential mates, whereas populations of dioecious species are reproductively subdivided into separate sexes, reducing the chance of encountering mates (Lloyd, 1982; Wilson and Harder, 2003). As a result, the reproductive success of sexual morphs should depend not only on density, but also on the sex ratio of reproductively mature individuals in the local mating neighborhood.

In dioecious populations, both density and sex ratio can influence the opportunity to mate with other compatible individuals through the actions of pollen vectors, typically small generalist insects or wind (Bawa, 1980; Wilson and Harder, 2003; Bianchi and Cunningham, 2012). Insufficient pollination may limit reproductive success in dioecious species (e.g., de Jong et al., 2005; Knight et al., 2005; but see Sutherland and Delph, 1984) and can be related to the demographic context of pollination in diverse ways, depending on the type of pollen vector (Wilcock and Neiland, 2002; Knight et al., 2005). For wind-pollinated species, pollination success may be influenced by density and sex ratio because pollen receipt depends on proximity to neighboring male plants (Steven and Waller, 2007; Hesse and Pannell, 2011; Compagnoni et al., 2017). This is caused by

distance-dependent dilution of pollen due to advection-diffusion dynamics of wind, gravitational settling of pollen, and filtration onto vegetative structures (Whitehead, 1969). As a result, mating in wind-pollinated species should occur less frequently among spatially distant individuals, and pollen limitation may be more evident at low density and in populations with female-biased sex ratios.

For insect-pollinated dioecious species, pollination success may not be as directly susceptible to distance-related pollen losses because pollen is carried between flowers on pollinators. Nonetheless, pollen limitation may be influenced by variation in the interactions between the behavior of pollinators and demographic factors (Ashman et al., 2004; Knight et al., 2005). The foraging behavior of insects is plastic, and individuals are likely to respond to the density and sex ratio of populations owing to variation in available floral rewards (Goulson, 1999). There is empirical evidence that dioecious populations occurring at low density are more pollen limited than high-density populations because they attract fewer reliable pollinators. Moreover, low density can promote more intensive within-plant foraging, thus limiting pollen dispersal among plants (Kay et al., 1984; House, 1992; Ashman et al., 2004; de Jong et al., 2005; Johnson and Nielson, 2014). Pollinators are usually more abundant and active in high-density populations because flight distances are shorter and foraging may be more energetically rewarding (Ghazoul, 2005; Essenberg, 2012).

Variation in sex ratio may also influence pollinator behavior because females do not typically produce pollen and are generally less rewarding than males, especially in nectarless species (Renner, 2006). Some pollinators (e.g., pollen-collecting bees and flies) discriminate against female flowers due to their lack of floral rewards (Bell et al., 1984; Kay et al., 1984; Ågren et al., 1986; Bierzychudek, 1987; Dukas, 1987; Kevan et al., 1990; Charlesworth, 1993; Ashman, 2000; Zhang and He, 2017), though not unequivocally (e.g., Borkent and Harder, 2007), and populations with a high frequency of less rewarding females can reduce the overall attractiveness of populations to discriminating pollinators (Ågren et al., 1986; House, 1993). Thus, as with wind pollination, populations at low density and/or that contain a high frequency of females may be more susceptible to pollen limitation (House, 1992; Knight et al., 2005; Voigt et al., 2005; Vamosi et al., 2006; Glaettli and Barrett, 2008).

Despite the potential importance of density and sex ratio for the reproductive success of dioecious plants, their combined effects have rarely been investigated experimentally (but see Van Drunen and Dorken, 2012; Compagnoni et al., 2017). In particular, it is unclear how such demographic factors might influence pollination dynamics in dioecious species that are ambophilous in which some populations may rely on both animals and wind for pollination. Ambophily is a relatively uncommon condition in flowering plants, and there is uncertainty as to whether it is an evolutionarily stable mixed strategy or an intermediate state in the evolutionary transition from insect to wind pollination (Culley et al., 2002; Friedman, 2011; Timerman and Barrett, 2018). If it is the former, then it might be considered a form of bet hedging if neither vector is consistently reliable owing to environmental heterogeneity in local habitat conditions. Although the predicted effects of density and sex ratio with animal and wind pollination may appear to be reinforcing, there is no reason to expect that they respond similarly to changes in either parameter. For example, one might envisage a scenario where increased plant spacing affects both vectors asymmetrically so that declining pollination success by one vector is compensated for by the other.

Here, we investigate the effects of density and sex ratio on pollinator behavior and reproductive success of females in the dioecious ambophilous herb *Thalictrum pubescens*. This species exhibits extensive variation in both density and sex ratio and inhabits many diverse moist habitats in eastern North America (Davis, 1997; Timerman and Barrett, 2019). Unlike most dioecious species, females produce stamens with infertile pollen (Davis, 1997). It is unclear why females maintain potentially costly stamens that do not apparently function directly in reproduction. One hypothesis is that they represent attractants for pollinators in otherwise rewardless females (Mayer and Charlesworth, 1991). However, Davis (1997) found that the occurrence of stamens in female *T. pubescens* did not influence female reproductive success, and instead proposed that a genetic constraint could be responsible for their maintenance (Davis, 2001). Without floral rewards, seed set in *T. pubescens* may be especially sensitive to demographic factors affecting the visitation rates of pollinators to female plants. In particular, we might expect visitation rates to females to vary significantly with flowering sex ratio. In male-biased patches, foraging would be more profitable and therefore one might expect a greater likelihood of insects "mistakenly" visiting female plants (see Wilson and Ågren, 1989). By contrast, in even or female-biased patches, we might expect a lower overall abundance of pollinators because foraging insects may learn to discriminate the less rewarding female sex. Similar arguments might also apply to density, where we might expect a reduction in visitation rates and foraging activity with decreasing density.

Using a field manipulative experiment involving arrays of *T. pubescens* varying in sex ratio and density, we addressed three questions: (1) Do pollinators display a preference for visiting male vs. female plants, and is there variation among specific pollinator groups (e.g., bees vs. flies) in whether they exhibited sex-specific preferences? We predicted that because females are nectarless and produce many fewer stamens than males, pollinators would prefer to visit males. (2) Does variation in density and sex ratio influence pollinator behavior? We were specifically interested in determining whether visitors spend more time foraging within individuals of a particular sex, and whether the choice to visit males or females was influenced by sex ratio and density. We predicted that pollinators would spend more time foraging within plants at lower density because flight distances are greater and moving between plants may be less profitable. (3) Does variation in both density and sex ratio affect female reproductive success? We predicted that in arrays with lower density and fewer males, there would be an overall reduction in seed set because both conditions are expected to reduce levels of insect and wind pollination.

MATERIALS AND METHODS

Study species and sampling

Thalictrum pubescens (Ranunculaceae), a perennial herb native to deciduous forests and wetlands of eastern North America (Kaplan and Mulcahy, 1971), is cryptically dioecious (a sexual system in which individuals of at least one sex are morphologically hermaphroditic but functionally unisexual; see Mayer and Charlesworth, 1991). It grows in both open and shaded habitats, in woods, thickets, marshes, meadows, ditches, and stream banks (Timerman and Barrett, 2019). Plants reproduce clonally by rhizomatous growth and sexually through ambophilous pollination (i.e., pollination by

both insects and wind; Timerman and Barrett, 2018). Flowering occurs from late June to early August, and populations are weakly protandrous. Floral displays are often large, consisting of hundreds to several thousand small, white flowers arranged in tall panicles up to 3 m in height (Fig. 1A, B), and can be up to 50% larger in males than in females (D. Timerman and S. C. H. Barrett, unpublished data). Flowers of both sexes are fragrant, produce no nectar or petals, and have small white sepals with exserted reproductive organs (Fig. 1C). The sexual system of *T. pubescens* is cryptic dioecy because female flowers always produce, in addition to several uniovulate carpels, functioning stamens with inapperturate (i.e., infertile) pollen (Fig. 1D), which may attract pollinators (but see Davis, 1997). Numerous stamens are the most conspicuous feature of male flowers but are four times fewer in number in female flowers. Although male flowers produce slightly longer stamens than female flowers, there is no sexual dimorphism in stamen mass, nutrient concentration, or pollen diameter (Davis, 2002). Populations exhibit extensive variation in flowering sex ratio but are male biased overall, with the bias likely established early in the life cycle, and not caused by sex-based differences in survival or flowering of mature genets (Timerman and Barrett, 2019).

In spring 2014, we excavated a random sample of 10–12 genets upon shoot emergence from each of nine populations across Ontario, Canada (for details, see Timerman and Barrett, 2018). We returned plants to a glasshouse at the University of Toronto, where they grew over two seasons in pots 25 cm in diameter, in

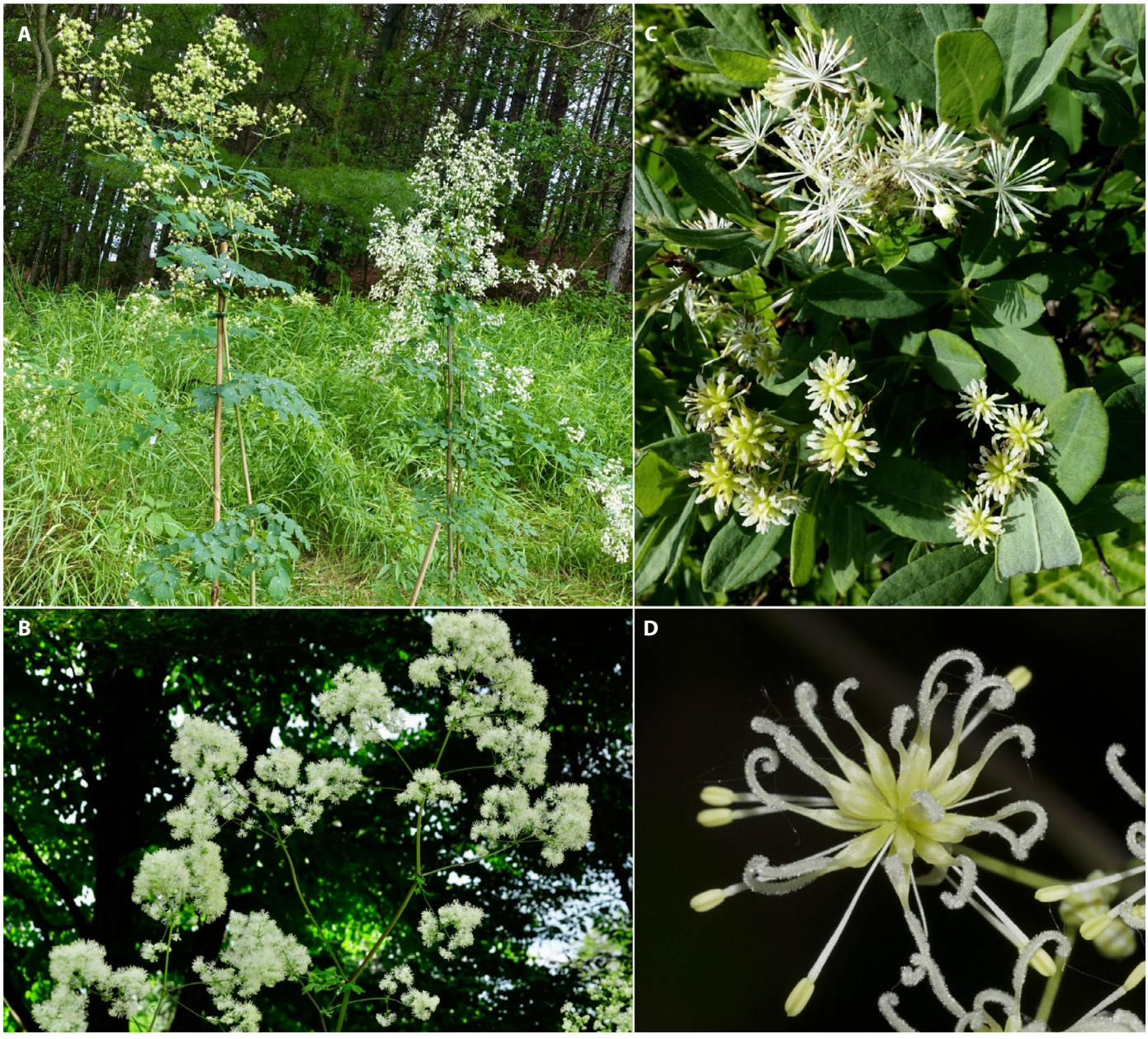

FIGURE 1. Flowers and floral displays of *Thalictrum pubescens*: (A) a female (left) and male (right) plant used in the experiment; (B) a male inflorescence; (C) clusters of female (bottom) and male flowers (top); and (D) a female flower with both stamens (outer whorl) and carpels (inner whorl). Image D courtesy of Stuart Campbell.

Pro-Mix BX soilless potting medium (Premier Tech, Rivière-du-Loup, Quebec, Canada). A subset of these genets (22 male and 18 female) was subsequently cloned in spring 2016 using four to six equally sized rootstock divisions. Each rootstock was placed individually in 25 cm pots with Pro-Mix. The clones were grown outdoors under shade cloth at the Koffler Scientific Reserve (KSR), Newmarket, Ontario (44°03′N, 79°29′W), and were fertilized every one or two weeks with a general-purpose fertilizer.

Experimental arrays

We investigated the influences of density and sex ratio of flowering individuals (hereafter "sex ratio") on pollination and seed set of *T. pubescens* using a manipulative field experiment. In summer 2017, we established replicated experimental arrays of eight plants varying both in density (low or high) and sex ratio (1:1 or 3:1 male-biased) at KSR. An extensive survey of 38 populations failed to reveal female-biased sex ratios in this species (Timerman and Barrett, 2019) and thus we chose to not investigate them in this study. In each array, we positioned plants randomly in an octagonal formation with edge lengths of 150 and 50 cm at high and low density, respectively. The arrays were located ≥100 m apart throughout the reserve along the edges of deciduous or mixed deciduous-coniferous woodlots and with intervening vegetation between arrays. The experiment comprised 24 arrays, with each combination of treatments replicated six times. For each array we measured plant height, ramet number, canopy type (closed or open), and date of peak flowering.

All plants of the same array were clones of a unique pairing of male and female genotypes to control for intrasexual differences in pollinator attraction and pollen dispersal and to ensure synchrony in the timing of flowering. Before the start of the experiment, we paired male (22) and female (18) genotypes based on their similarity in plant height, floral display size, and the developmental stage of floral buds (Appendix S1). Each pair was then randomly assigned a treatment combination and to one preselected field site where an array would be established. Most genotypes were used in only one array, but to achieve the desired level of replication, two male and six female genotypes were represented twice in the experiment. Arrays were set up with the requisite number of male and female clones to achieve a 1:1 or 3:1 sex ratio shortly before anthesis.

Pollinator visitation

We investigated the effects of experimental treatment on pollinator behavior first by quantifying visitation rates to female and male plants. We focused our effort on visits by hover flies (hf), solitary bees (sb), honey bees (hb), and bumble bees (bb) because they were the most common visitors (in order of visitation frequency) to *T. pubescens* over several consecutive years of observations at the field station (Timerman and Barrett, 2018). To estimate visitation rates, we counted the number of visitors of each type in a random sample of plants within arrays. Our observations were made over 16 days during 7–27 June 2017, when weather conditions were sunny or overcast but not raining. On each sampling day, we randomly chose up to seven arrays in which 75% (6/8) of plants were in peak bloom. Then, from 1000 to 1800 hours, we visited the arrays in random order, where two observers in tandem each counted the number of visitors to separate female and male plants in the same array, chosen and ordered at random, for 10 min each (totaling 40 min per array on each day).

In a separate set of observations taken during the same time interval, we quantified the duration of foraging visits to individual plants in a subset of arrays and tracked the movements of pollinators between plants. Our measurements were made on a random sample of four arrays per treatment except for the male-biased, low-density treatment for which we sampled three arrays. In each array, an observer walked slowly clockwise around the outer perimeter of the array until a visitor was spotted arriving at the plant nearest the observer. The observer then used a stopwatch to measure the time that the pollinator spent foraging on the focal plant and recorded the number of plants subsequently visited before departing from the array. This procedure was repeated for 30 min per array on random dates and times by two observers working concurrently in separate arrays.

Reproductive success of females

In September 2017, we sampled mature flowers from female plants to estimate the proportion of ovules setting seed (hereafter "seed set"). From two randomly chosen female plants per array, we sampled five clusters of 10 flowers haphazardly from throughout the floral display, and later counted the total number of mature seeds and unfertilized ovules. This procedure was relatively straightforward because *T. pubescens* has uniovulate carpels that rapidly swell upon fertilization. From these data we calculated the reproductive success (percent seed set) of females for each treatment.

Statistical analysis

To quantify the diversity of pollinators to males and females, we calculated the Shannon diversity index (Colwell, 2009), H, for each observation interval per plant. We then used two-tailed paired-sample t-tests on measurements of H averaged by array to evaluate whether pollinator diversity differed between the sexes.

Next, we applied generalized linear mixed models with Poisson errors (GLMM; Zuur et al., 2009) to evaluate the effects of sex, density, and sex ratio on pollinator visits per 10 min. As random effects, we included site (i.e., array identity) nested within days to account for repeated measurements within sites on different days, and a subject-level factor to account for overdispersion in our data. We repeated this analysis separately for each pollinator group to identify potential differences in their patterns of visitation.

We then used linear mixed models (LMM) to evaluate the effects of our treatments on the duration of foraging visits to plants of both sexes, and cumulative-link mixed models (CLMM) to determine whether the treatments affected the extent to which pollinators move between plants within arrays. In the LMM analysis, we included sex, density, and sex ratio as main effects and site as a random effect. Our response variable, duration, was log-transformed (base 10) to improve the model fit. Again, we separately analyzed each category of visitor in addition to modeling the pooled data set. We used the same random effect structure in the CLMM analysis, but as main effects included density, sex ratio, and pollinator type. Our response variable was the number of plants visited in sequence. We excluded bumble bees from these analyses due to a small sample size.

Finally, we evaluated the effects of our treatments on female reproductive success by modeling seed set as a function of density and sex ratio using a GLMM with binomial errors. Our response variable was binary given that each carpel has only one ovule that

can either be fertilized (1) or not fertilized (0), and was modeled for plants based on seed and unfertilized ovule numbers. As random effects, we included plants nested within site to account for repeated sampling within plants, and a subject-level factor to account for overdispersion in our data. We removed one outlier site from our analysis to ensure model convergence. The analysis was repeated to separately investigate the effects of the covariates plant height, ramet number, canopy type, and date of peak flowering.

For each analysis, we began with the most complex model, including two- and three-way interactions, and used likelihood ratios to drop higher-level terms that did not improve model fit, until the only remaining terms were significant individually or in an interaction term. All statistical analyses were performed using R version 3.5.2 (R Core Team, 2018) and the package lme4 (Bates et al., 2015).

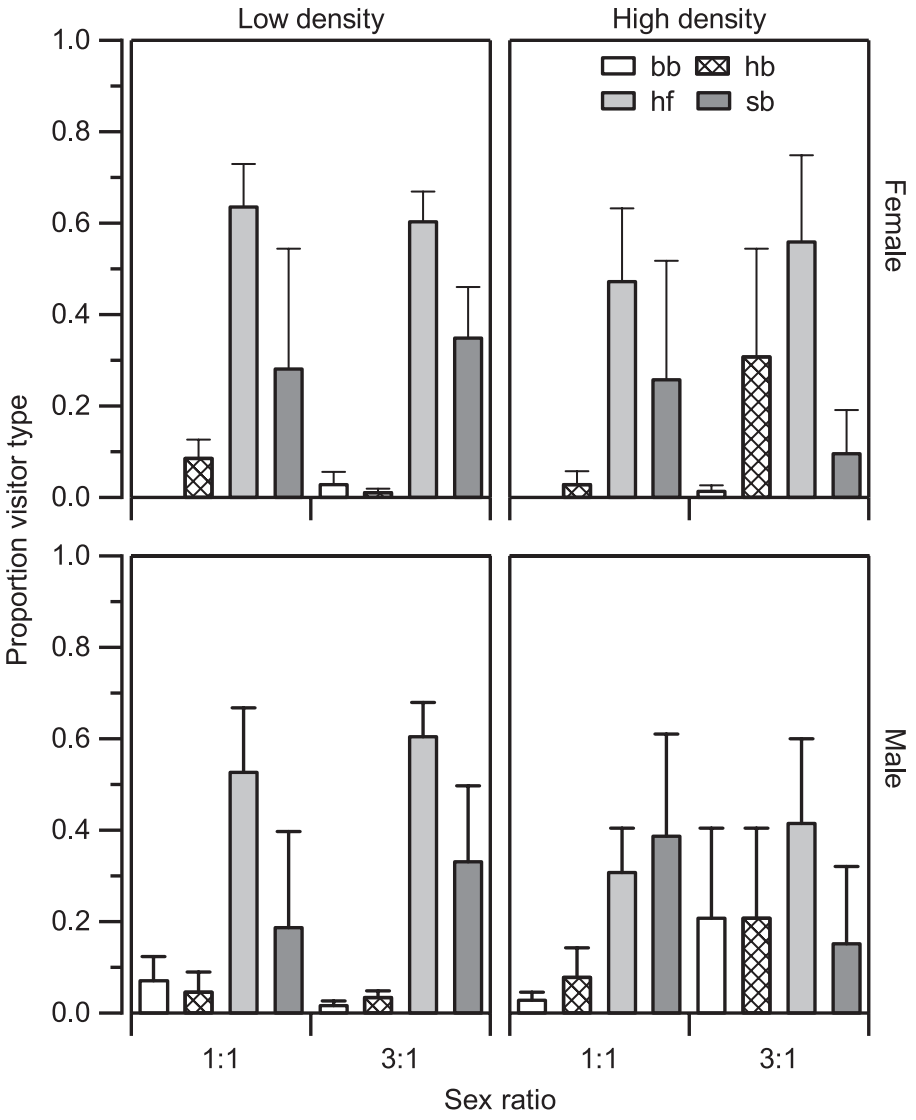

FIGURE 2. Proportions of visitor types to female and male plants of *Thalictrum pubescens* among arrays varying in density and sex ratio (male:female). Abbreviations: bb = bumble bees; hb = honey bees; hf = hover flies; sb = solitary bees. Error bars indicate standard error.

RESULTS

Visitation rates

Our observations of floral visitation spanned 42.7 h, during which time we recorded a total of 702 pollinators visiting our experimental arrays. The most common floral visitors were hover flies (Syrphidae, 62.3%); solitary bees (29.4%) of the families Andrenidae, Colletidae, and Halictidae; honey bees (*Apis mellifera*, 4.6%); and bumble bees (*Bombus* spp., 2.0%). Male plants showed a trend toward greater diversity in pollinators (males: $H = 0.70 \pm 0.08$, females: $H = 0.60 \pm 0.06$ [means ± SD]; Fig. 2), although the difference was not significant (paired *t*-test: $t = 1.40$, df = 18, $P = 0.089$). Male plants also received significantly more visits than females per 10 min time interval (likelihood ratio: $\chi^2_1 = 10.40$, $P = 0.001$; male: 3.2 ± 1.6, female: 1.9 ± 1.5; Fig. 3; Appendix S2). Hover flies and solitary bees maintained a constant level of visitation to female plants daily from 1000 to 1600 hours, but visitation to males peaked at ~1300 hours (Fig. 4). Bumble bee and honey bee visitation to males generally increased during this interval, and while this trend was mirrored for honey bee visits to females, bumble bee visits dropped precipitously. Further analysis of the patterns of sex-based differences in visitation by different pollinators revealed that solitary bees exhibited a clear preference for males ($\chi^2_1 = 11.27$, $P < 0.001$; Appendix S3). However, we found no overall difference in visitation to males and females by hover flies ($\chi^2_1 = 2.46$, $P = 0.117$). Our models also revealed no significant effects of density ($\chi^2_1 = 0.55$, $P = 0.459$) and sex ratio ($\chi^2_1 = 0.005$, $P = 0.941$) and no significant two- or three-way interactions between main effects (not shown) on overall visitation rates. Similar results were obtained in separate analyses of visitation by either solitary bees or honey bees.

Foraging behavior

We measured the duration of foraging visits for a total of 580 pollinators. Visitors spent significantly more time foraging on male plants (49.9 ± 4.1 s) than female plants (27.5 ± 2.9 s; Fig. 5; likelihood ratio: $\chi^2_1 = 8.98$, $P = 0.003$), irrespective of the density ($\chi^2_1 = 0.20$, $P = 0.654$; Appendix S4) and sex ratio ($\chi^2_1 = 0.07$, $P = 0.791$) of arrays. However, these differences between sexes were found only for honey bees ($\chi^2_1 = 7.37$, $P = 0.007$, $n = 121$; Appendix S5) and solitary bees ($\chi^2_1 = 5.03$, $P = 0.025$, $n = 279$; Appendix S6), and not hover flies ($\chi^2_1 = 5.03$, $P = 0.473$, $n = 163$). We did not separately evaluate the effect of sex for bumble bees because our sample size was too small (male plants: $n = 16$ visits; female plants: $n = 1$ visit). Despite the apparent interaction between

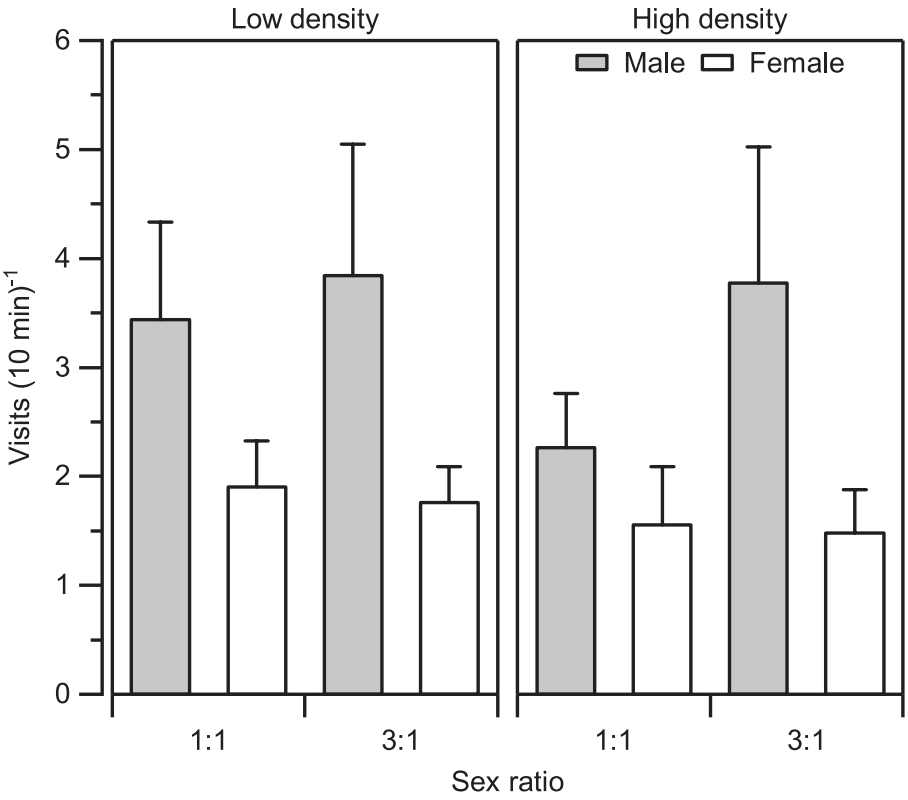

FIGURE 3. Average numbers of foraging visits by pollinators per 10 min interval to male and female plants of *Thalictrum pubescens* among arrays varying in density and sex ratio (male:female). Fixed effect of sex and two-way interaction of sex and sex ratio are significant at α = 0.05. Error bars indicate standard error.

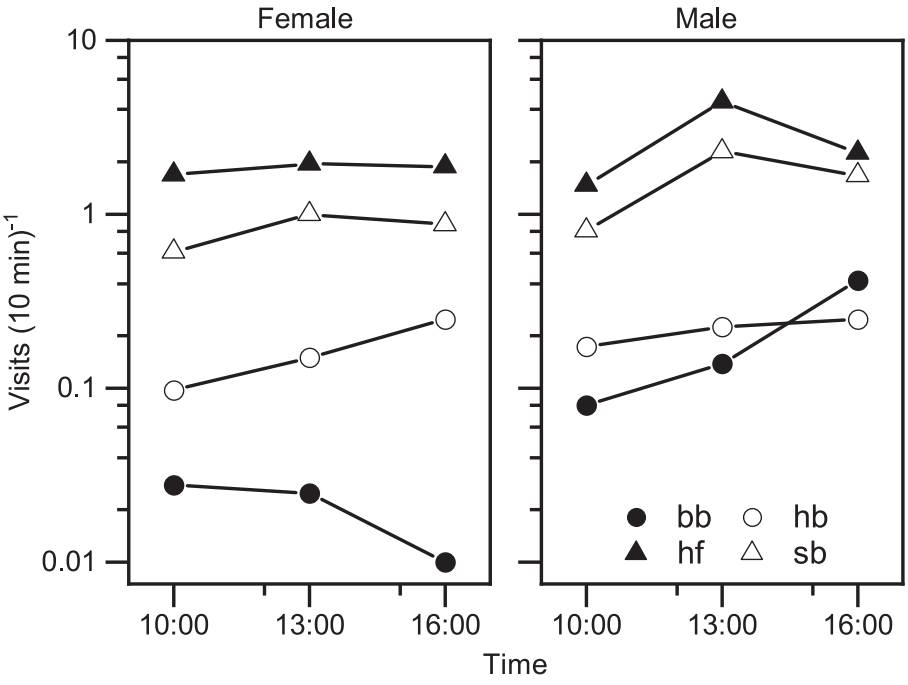

FIGURE 4. Daily average visitation rates to female and male plants of *Thalictrum pubescens* over time for different types of pollinators. Abbreviations: bb = bumble bees; hb = honey bees; hf = hover flies; sb = solitary bees.

pollinator type and sex, the main effect of pollinator type and its interaction with sex was not significant in the combined data set (excluding bumble bees; Fig. 5; Appendix S7).

Our CLMM model revealed that the number of plants visited during foraging bouts depended significantly on pollinator type ($P < 0.001$) and density ($P = 0.054$; Appendix S8), but not on their interaction ($P < 0.526$; Fig. 6). In the observed sequences of visits, honey bees visited the most plants (2.29 ± 1.26 plants, $n = 121$), followed by solitary bees (1.78 ± 0.96 plants, $n = 279$) and hover flies (1.34 ± 0.72 plants, $n = 163$). Pollinators also visited a greater number of plants while foraging in high-density arrays (1.89 ± 1.11 plants, $n = 312$) compared to low-density arrays (1.61 ± 0.89 plants, $n = 251$). We detected no significant effects of sex ratio ($P < 0.981$) or its interaction with other fixed factors (data not shown).

Female reproductive success

Our analysis of female reproductive success revealed a significant interaction between density and sex ratio ($\chi^2_1 = 4.64$, $P = 0.031$; Appendix S9), with seed set significantly higher at 3:1 than at 1:1 sex ratio at low density, but with the opposite trend at high density (Fig. 7). There were no significant effects of the covariates plant height, ramet number, canopy type, or date of peak flowering on seed set (data and analysis not shown).

DISCUSSION

The objective of our study was to determine how demographic variation influences pollinator behavior and mating success in a species with ambophilous pollination. Our investigation of *T. pubescens* revealed interactive effects of plant density and sex ratio on seed set, which could not be explained by the foraging behaviors of insect pollinators. Despite an overall preference of solitary bees for foraging on male plants, visitation rates and foraging times did not depend on demographic factors for any pollinator group. Moreover, whereas density influenced the tendency for pollinators to move among plants, seed set was similar among the density treatments. Several factors may explain these results,

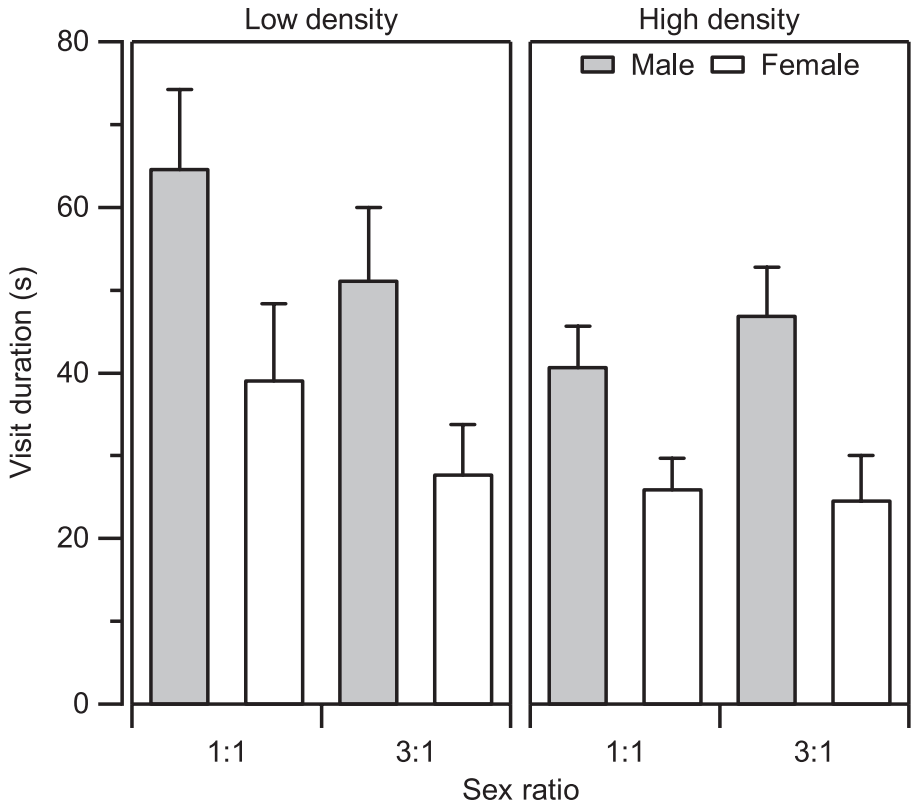

FIGURE 5. Average durations of foraging visits by pollinators to male and female plants of *Thalictrum pubescens* among arrays varying in density and sex ratio (male:female). Fixed effect of sex is significant at α = 0.05. Error bars indicate standard error.

including the influence of density and sex ratio on pollen export from arrays, on grooming by pollinators, and on variation in the contribution of wind pollination.

Insect pollinators differ in sex-specific foraging preferences

The rate of insect visitation to flowers varied between the sexes, but in tests of individual pollinator groups the difference was only significant for solitary bees. Nevertheless, all four pollinator groups generally displayed greater rates of visitation to males at most times of the day (see Fig. 4). Male plants were also visited for longer durations compared to female plants (Fig. 5). These results supported our prediction that pollinators would display sex-specific foraging preferences, but with the caveat that these patterns were more accentuated for solitary bees. Our findings are also consistent with the observation that emasculated female flowers of *T. pubescens* received fewer pollinator visits than intact flowers (Davis, 1997), presumably because pollinators are attracted to the presence of pollen.

Another explanation for the foraging bias of solitary bees compared to hover flies is that increased pollinator activity on male plants by bees induced hover flies to forage more on female plants. Few studies have investigated competitive interactions between bees and flies in pollination (but see Morse, 1981; Inouye et al., 2015), but in cryptically dioecious *Rosa setigera*, hover flies switched from male to female plants as bees became prevalent on males, potentially reducing interspecific competition for pollen (Kevan et al., 1990). However, our data do not support this hypothesis, because hover flies visited females at a consistent rate and their diurnal patterns of visitation to males was similar to that of solitary bees (Fig. 4). In addition, the overall abundance of both pollinator groups was quite low, thus limiting opportunities for competitive interactions.

The observed differences in selectivity may also be due to cognitive differences between the two pollinator groups. Unlike most bees, adult hover flies do not collect pollen for larval provisioning and instead only visit flowers to feed (Thorp, 2000; Woodcock et al., 2014). This contrast in activity may necessitate more available pollen being required for bees than for flies and, hence, bees' preference for visiting male flowers. Foraging on females may therefore incur a reduced cost in hover flies compared to pollen-collecting bees. The two groups of pollinators may also differ in their abilities to perceive the contrasting pollen rewards offered by male and female plants. Species from both pollinator groups, however, often exhibit well-developed abilities to learn olfactory and visual stimuli that signal pollen availability, resulting in discriminating behaviors (Lunau, 2000; Raguso, 2008; Wright and Schiestl, 2009; Woodcock et al., 2014; Nicholls and Hempel de Ibarra, 2017). To our knowledge, no study has compared the relative responses of bees and flies to pollen availability in male and female plants of a dioecious species. Therefore, it remains unclear to what extent cognitive differences between bees and flies may have contributed to the sex-specific foraging preferences that we observed.

Our results contribute to the growing body of evidence that pollinators of dioecious species prefer visiting male plants (Bell et al., 1984; Kay et al., 1984; Ågren et al., 1986; Bierzychudek, 1987; Dukas, 1987; Kevan et al., 1990; Charlesworth, 1993; Ashman, 2000; Zhang and He, 2017). This sex-specific difference in foraging raises questions concerning the importance of pollinator selectivity in the evolution of dioecy (Ashman, 2000; Borkent and Harder, 2007). It has been argued that pollinator selectivity may represent an important constraint on the evolution of dioecy if females suffer reduced fertility, compared to ancestral hermaphrodites, due to their lack of floral rewards (Baker, 1976; Lloyd, 1982). Significantly, many dioecious species, including *T. pubescens*, are pollinated by small, generalist insects that are considered opportunistic foragers (Bawa, 1994; Zhang and He, 2017). Charlesworth (1993) proposed that the reason for this association could be that these pollinators visit plants of both sexes indiscriminately, thus removing the selective barrier of sex-specific foraging preferences. However, results from several studies (see above), including our own, contradict this prediction in that sex-specific pollinator selectivity has often been observed in dioecious species. But pollinator selectivity may have other important consequences for the evolution of dioecy. For example, sex-specific foraging may constrain the sexual morphs of dioecious species from becoming too divergent in attractive traits and

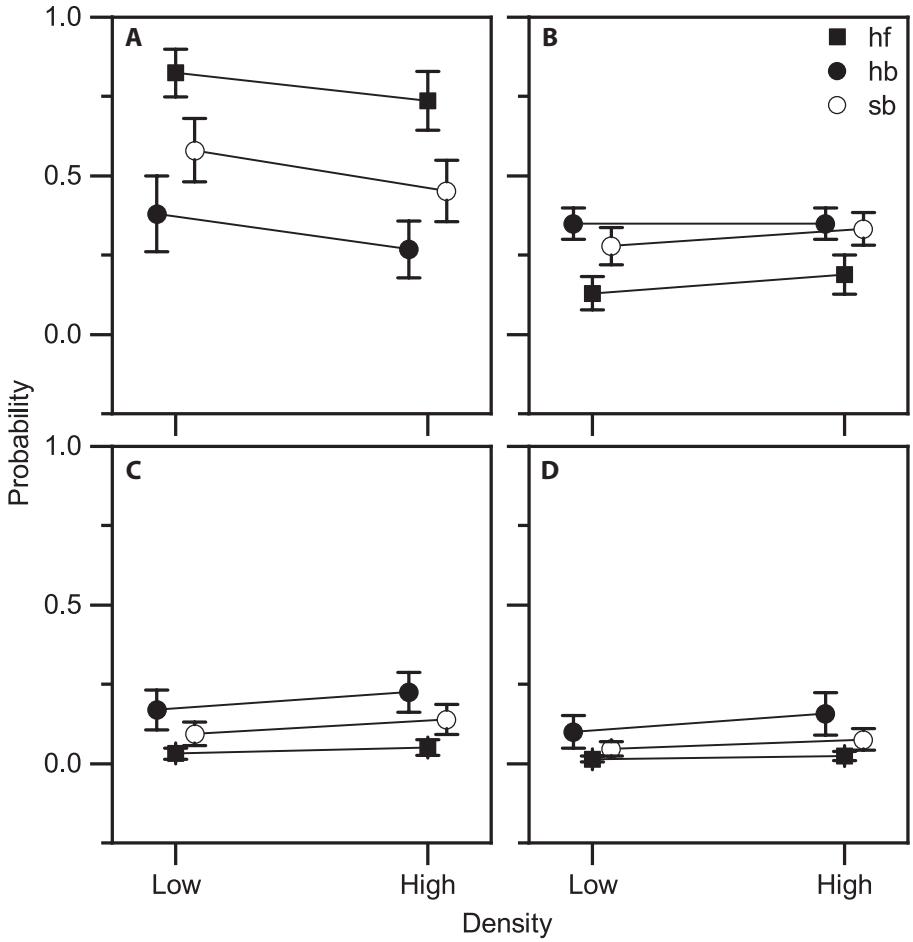

FIGURE 6. Results of the cumulative-link mixed-model analysis for the number of plants visited by hover flies (hf), honey bees (hb), and solitary bees (sb) during foraging bouts on arrays of *Thalictrum pubescens*. The y-axis represents the predicted probability that individual pollinators visit one (A), two (B), three (C), or greater than three (D) plants before departing the array. Significant main effects were detected for both density (low and high) and pollinator type, but not for their interaction (see text for details).

rewards (Ashman, 2000) and may even promote the evolution of floral characters in females that mimic the appearance of male flowers (Willson and Ågren, 1989). This phenomenon termed "deceptive pollination" could explain the occurrence of stamens in females of *T. pubescens* because they function to increase visitation to females (Davis, 1997).

Effects of demographic variation on foraging patterns

Despite our initial predictions, we found no significant effects of density and sex ratio on visitation rates and foraging times of pollinators. However, we did find a significant positive effect of density on the number of plants visited during individual foraging bouts (Fig. 6). Whereas a lack of power due to small sample size may explain our equivocal results, several other factors could have contributed to this outcome. Because our arrays were distributed over a wide spatial area (80 ha), which may often have exceeded the typical flight distances and foraging ranges for small bees and flies (Zurbuchen et al., 2010; Inouye, 2015), individual pollinators likely encountered only a single or small subset of the total arrays and thus were not "choosing" among treatments. Moreover, the increased distance separating plants in low-density arrays compared to high-density arrays may not have been large enough to elicit substantial changes in foraging behavior. Local density effects also depend on the presence or absence of other co-occurring flowering species and the spatial scales at which pollinators perceive patchiness (Thomson, 1981; Klinkhamer et al., 2001). However, most of our arrays were bordered by mown fields and woodlots, so there were few co-flowering species, most of which had much smaller floral displays compared to those of *T. pubescens*. It is likely, then, that our arrays were the most rewarding resource patches available at their respective sites, and their presence may have outweighed any influence of density and sex ratio on visitation rates.

We expected that pollinators would spend more time foraging within plants at low density and 1:1 sex ratio because of increased travel costs between plants combined with a preference for visiting males. There are indications that this prediction was realized, in that average foraging time on plants for both sexes was greatest for the arrays with low density and 1:1 sex ratio (Fig. 5). However, the overall lack of significance for both density and sex ratio suggests that these effects were generally weak or absent, implying that our treatments did not greatly influence foraging costs.

Factors influencing female reproductive success

Overall levels of seed set (fruit set) in our arrays averaged 56%, a value significantly lower than the average reported for dioecious species (see Sutherland and Delph, 1984) but in accord with levels previously found in several investigations of open-pollinated seed set in *T. pubescens* (46–70%; Kaplan and Mulcahy, 1971; Melampy and Hayworth, 1980; Davis, 2004). We found a significant crossover in the interaction between density and sex ratio on seed set, indicating that the demographic context for pollination was influential in determining female reproductive success (Fig. 7; Appendix S9). The crossover interaction was unexpected given our initial prediction that density and sex ratio would have positive additive effects on seed set for both pollen vectors. We further expected our predictions to be realized, given that overall pollinators spent more time collecting pollen in 3:1 than in 1:1 arrays (i.e., because there were more males to visit in 3:1 arrays) and visited more plants at high density than at low density, both of which should have resulted in greater pollen transfer among plants. However, there was a 14% reduction in seed set for 3:1 compared to 1:1 arrays at high density, which was opposite to our predictions (Fig. 7, right panel). It seems unlikely that wind pollination would have contributed to reduce seed set in 3:1 arrays, because

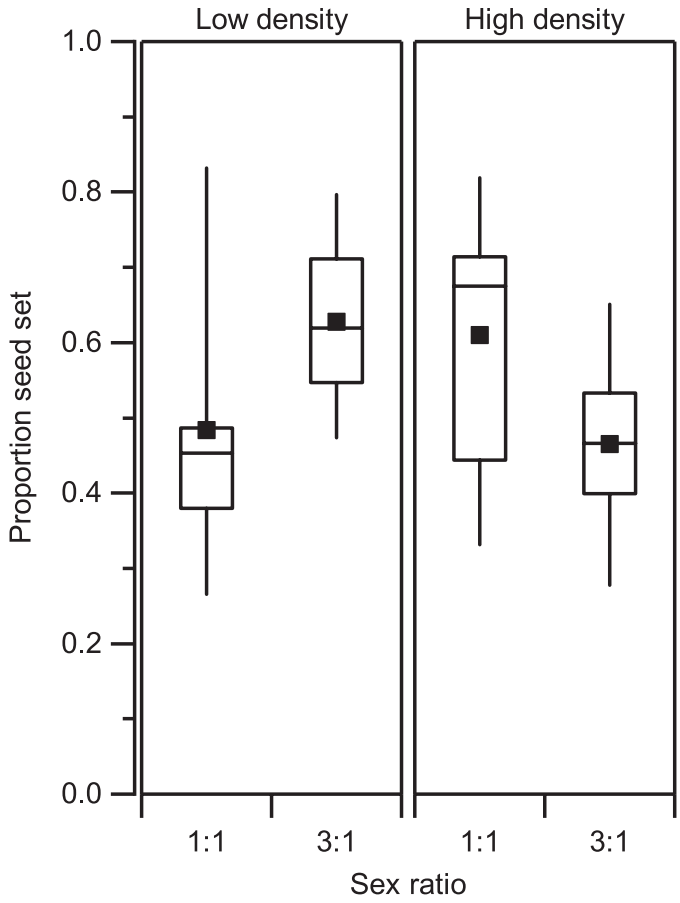

FIGURE 7. Box plots of the proportion of seed set for arrays of *Thalictrum pubescens* varying in density and sex ratio (male:female). The top and bottom of boxes indicate the 75th and 25th quartiles, respectively; whiskers indicate the minimum and maximum values; the middle line indicates the median; and filled squares indicate the mean. A significant effect of density × sex ratio interaction was detected at $\alpha = 0.05$.

the only conceivable effect of increasing male density is increased pollen transfer to females; that is, seed set is usually regarded as an increasing function of pollen production and density (production per area) in wind-pollinated species (Schoen and Stewart, 1986; Allison, 1990; Smith et al., 1990; Cox, 1991; Friedman and Barrett, 2009; Nakahara et al., 2018; Tonnabel et al., 2019). Thus, it is unclear why seed set was reduced in male-biased arrays compared to those in which the sexes were equally represented.

We expected that the combined effect of high density and 3:1 sex ratio would be greater pollen transfer by insects, but our results for seed set suggest that the opposite occurred. This implies that pollinators may actually have transferred fewer pollen grains to stigmas. One possibility is that a greater frequency of males induced pollinators to groom more often because they would become saturated with pollen more frequently (Harder, 1990; Thorp, 2000). This could have resulted in a reduction in pollen available to be dispersed by either insects or wind. A similar effect may not have been observed at 1:1 sex ratio because individual pollinators visited fewer plants on foraging bouts. Unfortunately, we do not have data on pollen loads on pollinators and stigmas, which could be used to determine if fewer pollen grains were delivered as a result of pollinator grooming.

Because wind pollination is a distance-dependent process (Whitehead, 1969), we expected seed set to decrease at low density in comparison to high density. Despite our prediction, we found no overall difference in seed set between densities (54% vs. 56% seed set; see Fig. 7). This could indicate that wind pollination was not particularly sensitive to increased plant spacing or that insect pollination was effective at maintaining seed set. We consider the second possibility more plausible given that wind pollination has consistently been found to decrease with distance from a pollen source (Di-Giovanni and Kevan, 1991; McCartney, 1994; Vekemans and Hardy, 2004; Ghazoul, 2005), including in two wind-pollinated species of *Thalictrum* (Steven and Waller, 2007). Interestingly, our results for seed set at high density were reversed at low density (Fig. 7, left panel); seed set was greater at 3:1 than at 1:1 sex ratio, as we initially predicted it should be. A variety of post-pollination processes, including gametophytic competition, selective abortion, and resource allocation, also contribute to variation in the female fertility of plants (Stephenson, 1981; Harder and Routley, 2006; Stehlik and Barrett, 2006). Without additional information it is difficult to speculate on the mechanisms responsible for the differences we recorded in the seed set of arrays.

Why is ambophily rare?

Our results reveal a complex interplay between density and sex ratio on seed production, likely mediated, in part, through their effects on pollination. Although it was not technically feasible to quantify the relative contributions of insect and wind pollination to reproduction, our results suggest that they respond differently to changes in density and sex ratio. Assuming that wind pollination was more effective at high than at low density, as reported for many dioecious species (e.g., Allison, 1990; Knapp et al., 2001; Steven and Waller, 2007; Hesse and Pannell, 2011; Compagnoni et al., 2017), the net contribution of insect pollination to seed production must have been positive at low density, given that seed set was relatively consistent among density treatments (Fig. 7). This suggests that ambophily may be an especially important pollination system in variable pollination environments caused by heterogeneous ecological and demographic conditions.

If ambophily indeed represents a functionally flexible pollination strategy, it raises questions as to why it is apparently relatively uncommon among angiosperms (see Culley et al., 2002). One explanation is that ambophily may occur more frequently than is reported, and indeed an increasing number of species have been described as ambophilous (Stelleman, 1984; Berry and Calvo, 1989; Vroege and Stelleman, 1990; Gomez and Zamora, 1996; Goodwillie, 1999; Totland and Sottocornola, 2001; Culley et al., 2002; Lázaro and Traveset, 2005; Gulías and Traveset, 2012; Yamasaki and Sakai, 2013; Rios et al., 2014; Wang et al., 2017; Rosado et al., 2018; Saunders, 2018). A second possibility is that ambophilous phenotypes may have reduced fitness compared to individuals adapted for a single pollination strategy. Insect and wind pollination require strikingly different floral adaptations to function effectively, and thus structural and functional compromises may be necessary for ambophily to be maintained. Such compromises may potentially reduce the effectiveness of either pollen vector, resulting in lowered fitness. For example, a conflict exists between the possession of attractive petals and the capture of airborne pollen in insect-pollinated *Brassica napus*, hindering ambophily (Cresswell et al., 2004). Transitions from insect

to wind pollination may require consistently strong selection for species to cross this potential fitness valley, and ambophily may often be too evolutionarily transient to observe in lineages where wind pollination has evolved.

For ambophily to spread and be maintained for long enough to observe, it must confer a fitness advantage to balance its costs compared to the "pure" strategies of animal and wind pollination. A resolution to this potential low-fitness problem is bet hedging (reviewed in Simons, 2011) with respect to pollen dispersal mechanism. Although plant fitness may be maximized by specializing on a single functional group of pollen vectors, wide-ranging and ecological versatile species that inhabit diverse environments, such as *T. pubecens*, may accrue long-term benefits from ambophily because animal pollination may not be consistently more reliable than wind pollination across space and time.

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AUTHOR CONTRIBUTIONS

D.T. and S.C.H.B. conceived the ideas and designed the experiments. D.T. collected the data and wrote the manuscript. Both authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY

Data are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.79cnp5hrs (Timerman and Barrett, 2020).

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

APPENDIX S1. Mean ± standard deviation of plant traits for arrays of *Thalictrum pubescens* in the experiment.

APPENDIX S2. Summary of generalized linear mixed model (Poisson errors) describing variation in insect visitation rate (bumble bees, honey bees, hover flies, and solitary bees) to plants in arrays of *Thalictrum pubescens*.

APPENDIX S3. Summary of generalized linear mixed model (Poisson errors) describing variation in visitation rate by solitary bees to plants in arrays of *Thalictrum pubescens*.

APPENDIX S4. Summary of linear mixed model describing variation in log visit duration of insects (bumble bees, honey bees, hover flies, and solitary bees) to plants in arrays of *Thalictrum pubescens*.

APPENDIX S5. Summary of linear mixed model describing variation in log visit duration of honey bees to plants in arrays of *Thalictrum pubescens*.

APPENDIX S6. Summary of linear mixed model describing variation in log visit duration of solitary bees to plants in arrays of *Thalictrum pubescens*.

APPENDIX S7. Summary of linear mixed model describing effects of sex, visitor type, and their interaction on variation in log visit duration of insects (honey bees, hover flies, and solitary bees) to plants in arrays of *Thalictrum pubescens*.

APPENDIX S8. Summary of cumulative-link mixed model describing the probability that individual pollinators visited one, two, three, or more than three plants in arrays of *Thalictrum pubescens*.

APPENDIX S9. Summary of generalized linear mixed model (binomial errors) describing variation in proportion of seed set among female plants in arrays of *Thalictrum pubescens*.

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