

Small and surrounded: population size and land use intensity interact to determine reliance on autonomous selfing in a monocarpic plant

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- **Background and Aims** Habitat fragmentation has transformed landscapes globally, leaving remnants embedded within a complex matrix that is rapidly becoming more developed. For many plant populations, the associated factors of decreased size and intensification of land use surrounding them are expected to increase pollen limitation ('PL'), unless autonomous self-pollination provides reproductive assurance ('RA'). Decreased pollinator visitation is often assumed to drive these patterns, but other, less studied mechanisms might include increased heterospecific pollen transfer or decreased conspecific pollen availability via florivory. I investigate how PL and RA and their potential underlying mechanisms vary with population size and land use intensity surrounding populations in the biennial *Sabatia angularis* (Gentianaceae).
- **Methods** I estimated the capacity for seed production via autonomous self-pollination (i.e. autofertility). Over 2 years in 22 *S. angularis* populations across a fragmented landscape, I performed emasculation and pollen supplementation experiments measuring RA and PL, and quantified visitation rates of potential pollinators and a pollen consumer, conspecific pollen loads and rates of heterospecific pollen deposition.
- **Key results** Autofertility based on fruit mass was 93 % under PL but only 51.6 % relative to maximal conditions. PL and RA were significant on average across populations in the first year of study. Variation in RA was significantly influenced by the interaction between population size and land use intensity, which in turn rendered PL independent of these factors. Visitation and heterospecific pollen deposition rates were greatest in small populations and declined with population size, while conspecific pollen loads were greatest in intermediate sized populations.
- **Conclusions** Increased reliance on RA is predicted in small *S. angularis* populations surrounded by intense development, which can explain elevated selfing rates in fragmented populations of plant species more generally. Results from this study point toward forces such as heterospecific pollen transfer, self-pollen limitation or resource availability influencing the need and ability to rely on RA.

Key words: Autofertility, autonomous selfing, florivory, habitat fragmentation, heterospecific pollen transfer, land use intensity, plant reproduction, pollen deposition, pollen limitation, pollinator visitation, reproductive assurance, *Sabatia angularis*

INTRODUCTION

Globally, intensification of human land use from conversion of native habitats to agriculture, housing and other forms of development is rapidly changing the landscape (Foley *et al.*, 2005; Hooke *et al.*, 2012). A major concern for wild plant populations is how these changes will impact plant–pollinator interactions and subsequent reproduction (Rathcke and Jules, 1993; Kearns *et al.*, 1998; González-Varo *et al.*, 2013). Given that nearly 90 % of angiosperms rely on biotic pollination for successful seed production (Ollerton *et al.*, 2011), understanding how pollination dynamics change with local- and landscape-level impacts of land use is critical for the conservation of remnant plant populations.

For many plant species, the degree to which seed production is limited by compatible pollen receipt, i.e. pollen limitation ('PL'), is expected to increase in response to both local- and landscape-level metrics of human land use, including reduced population size and increased fragmentation or land use

intensity (Ashman *et al.*, 2004; Knight *et al.*, 2005). In fact, one meta-analysis concluded that PL is probably the 'most proximate cause of reproductive impairment in fragmented habitats' (Aguilar *et al.*, 2006), which, if sustained, could lead to lower recruitment and even population growth (Ashman *et al.*, 2004). Yet, in comparison with studies of plant population size on pollinator visitation or mean seed production, few have explicitly measured the effect of population size on PL in wild populations (reviewed in Knight *et al.*, 2005). Further, despite the fact that fragmented populations are often nestled in a complex landscape matrix, the impact of surrounding land use and its interaction with population size on pollination dynamics remains relatively unexplored (Jules and Shahani, 2003; Hadley and Betts, 2012).

For some wild plant species, PL may be abated through autonomous self-pollination (i.e. self-pollination in the absence of pollinators) providing reproductive assurance ('RA') (Schoen and Brown, 1991; Kalisz and Vogler, 2003; Eckert *et al.*, 2006). Numerous plants are capable of autonomous self-pollination,

and for some this ability boosts seed production, a minimum requirement of the RA hypothesis (Busch and Delph, 2012; Ruan and Teixeira da Silva, 2012). Less is known about the extent to which the level of RA varies in relation to ecological context (Kalisz *et al.*, 2004; van Kleunen *et al.*, 2007; Jacquemyn and Brys, 2008; Brys *et al.*, 2011). Such investigations are necessary, because although self-compatible species are generally less likely to experience PL (Burd, 1994; Larson and Barrett, 2000), not all self-compatible species are capable of autonomous selfing, and this ability is not necessarily correlated with RA (Ruan and Teixeira da Silva, 2012). Moreover, examinations of the influence of the surrounding landscape matrix on RA are virtually non-existent (but see Brys and Jacquemyn, 2012) despite its links to pollinator and plant abundance. Increased RA with declines in population size and/or land use intensity would explain, in part, why reproduction in self-compatible species is less likely to be affected by fragmentation than self-incompatible species (Aguilar *et al.*, 2006) and why selfing rates are elevated in many small, fragmented and/or disturbed populations (Aguilar *et al.*, 2008; Eckert *et al.*, 2010).

Pollen limitation and reliance on RA are symptomatic of receiving either insufficient amounts of conspecific pollen or excessive heterospecific pollen. Lower conspecific pollen loads are expected because of reduced pollinator visitation rates. Where fragmentation and habitat loss have led to local reductions in plant population size, populations may be rendered less attractive to foraging pollinators (Sih and Baltus, 1987; Jennersten, 1988; Kunin and Shmida, 1997). Compounding this problem, greater development surrounding fragments can lead to decreases in floral resources and/or pollinator nesting sites, reducing pollinator abundance and richness (Carvell *et al.*, 2006; Kremen *et al.*, 2007; Clough *et al.*, 2014; Baude *et al.*, 2016). However, fragmentation may also alter antagonistic interactions (Chávez-Pesqueira *et al.*, 2015) that could also influence conspecific pollen loads. In particular, a relatively untested hypothesis is that reduced conspecific pollen loads arise because of increased pollen consumption by florivores in small populations or those most impacted by land use (Malo *et al.*, 2001). In fact, the impact of pollen consumers on PL and RA more broadly is largely unknown (Hargreaves *et al.*, 2009).

Little is also known about the effects of human land use on heterospecific pollen transfer. However, habitat fragmentation or loss with subsequent changes in population size and the landscape matrix can influence pollinator composition and plant-pollinator network structure (reviewed in Hagen *et al.*, 2012). In particular, studies have found that habitat loss and intense land use lead to the loss of efficient pollinators and increased pollinator generalization (Larsen *et al.*, 2005; Theodorou *et al.*, 2017). Consequently, increased heterospecific pollen transfer might arise with decreased population size and/or increased land use intensity. Heterospecific pollen transfer can lead to reduced fecundity by physically clogging stigmas or interfering with conspecific pollen performance (Morales and Traveset, 2008). Recently, the influence of heterospecific pollen transfer on selfing has also been recognized (Arceo-Gómez and Ashman, 2014; Brys *et al.*, 2016). Because autonomous self-pollination could alleviate heterospecific pollen effects if it occurred prior to heterospecific pollen arrival or dilute the effects if it increased the relative conspecific pollen load, greater reliance on autonomous self-pollen (i.e. RA) may evolve where

heterospecific pollen transfer is greater (Goodwillie and Ness, 2013; Brys *et al.*, 2016).

Here, I investigate how PL and RA and their potential underlying mechanisms vary with population size and surrounding land use intensity across wild populations of the self-compatible biennial *Sabatia angularis*. Previous work identified the importance of population size on reproductive patterns in this species, demonstrating that small populations experience reproductive Allee effects (Spigler and Chang, 2008) and increased selfing rates (Spigler *et al.*, 2010), but the underlying mechanisms are unknown and the role of surrounding land use intensity has not yet been explored. I present a series of studies conducted over 2 years and 22 *S. angularis* populations occurring in remnant serpentine barren grassland patches across a complex landscape. I first confirm that plants can autonomously self-pollinate and quantify the fraction of seed production that can be achieved through autonomous self-fertilization (i.e. autofertility). I then ask the following questions. (1) Is seed production across wild populations pollen limited, and does autonomous selfing provide RA? (2) To what extents do population size, surrounding land use intensity, measured as the proportion of developed land within 0.5 km of populations, or their interaction influence PL and/or RA? To address the underlying mechanisms, I further asked, how do (3) visitation rates by potential pollinators and a pollen consumer and (4) conspecific and heterospecific pollen deposition vary with population size and/or surrounding land use intensity?

MATERIALS AND METHODS

Study system

Sabatia angularis (L.) Pursh (Gentianaceae) is a herbaceous biennial found in a variety of open habitats (glades, old fields, roadsides and grasslands) throughout eastern North America. Plants produce showy, pink flowers from July–August that are visited by a suite of generalist pollinators for a pollen reward and develop into many seeded, dry dehiscent capsules in the autumn. *Sabatia angularis* is self-compatible and on average is mixed mating (multilocus outcrossing rate $t_m = 0.78 \pm 0.12$ s.d.), but primary selfing rates ($1 - t_m$) can reach nearly 50% (Spigler *et al.*, 2010, 2017). Population size explains a large proportion of the variation in selfing ($R^2 = 0.53$), with elevated selfing rates in small populations (Spigler *et al.*, 2010). The predominant mode of self-pollination is unknown. Pollinator-mediated selfing is possible given that plants can simultaneously present >100 open flowers (mean 14 ± 13.6 s.d. flowers, $n = 602$ plants), but autonomous selfing is also possible when male and female phases of the protandrous flowers overlap. The mechanism of autonomous selfing is unknown, as is the degree of autofertility and the extent to which it provides RA in this species.

This study took place across *S. angularis* populations in the rare serpentine grasslands of south-eastern Pennsylvania, USA (Fig. 1). These unique habitats have undergone dramatic declines in the last century (Latham, 1993). Urban development, mining and changes in historical fire and grazing regimes have all led to habitat loss, with extirpation of entire sites and reduced extent of remaining sites. Historically situated in a matrix of mixed hardwood forest, these imperiled habitats are

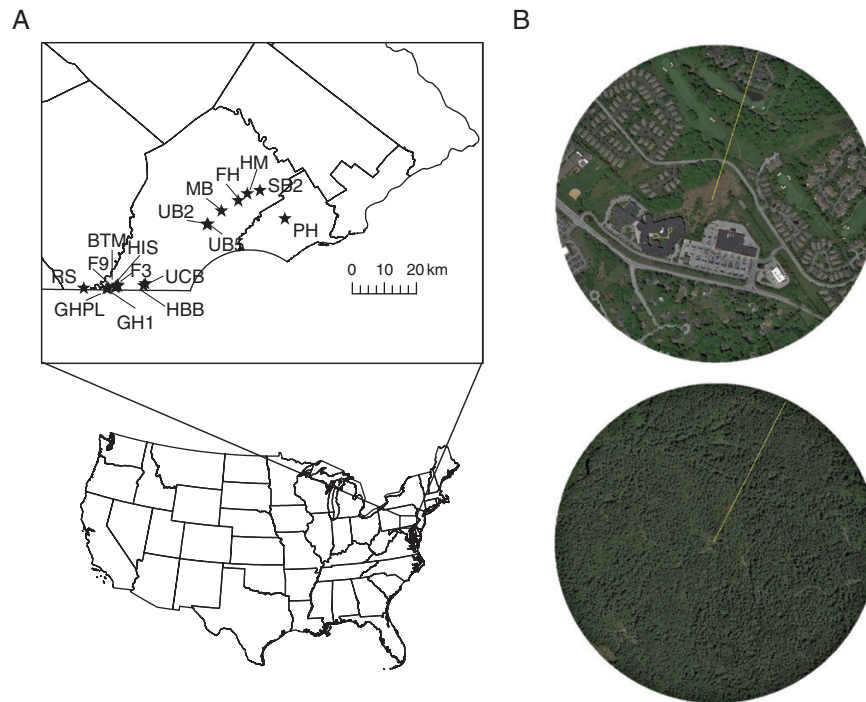


FIG. 1. Map of *Sabatia angularis* study sites. (A) Regional map showing the location of study sites across counties in south-eastern Pennsylvania, USA. (B) Google Earth images (Map data: Google 2016) illustrating the extremes of surrounding land use intensity within a 0.5 km radius of populations at two sites (HM, top, and BTM, bottom). See Supplementary Data Table S1 for a complete list of sites.

now embedded in a patchwork of agricultural, residential and industrial development. Consequently, remnant grasslands vary in the land composition surrounding them, and *S. angularis* populations occurring in these grasslands vary greatly in size. The present study was conducted over 2 years. Because of its biennial life cycle, *S. angularis* adults are not present every year in all sites. In 2014, I identified and included nine *S. angularis* populations. In 2015, 13 populations were included, six at sites used in 2014 and seven representing new serpentine sites. Thus, a total of 22 populations across both years were investigated (see Supplementary Data Table S1 for population details).

Characterizing population size and surrounding land use intensity

Population size was determined either by directly counting all flowering plants or, in the largest populations, from transects. In the latter case, population size was extrapolated based on transect-based estimates of plant density and grassland area, calculated in ArcGIS 10.2.2 using GPS waypoints taken along each grassland perimeter.

The composition of land use surrounding each population was quantified using ArcGIS 10.2.2 and data gathered from the 2014 Cropscape (USDA National Agricultural Statistics Service) data set at a spatial resolution of 30 m and projected to NAD 83 UTM zone 18. I defined and quantified land use intensity as the cumulative proportion area of high-, medium- and low-intensity and open developed spaces, representing various residential and industrial uses, within 0.5 and 1 km radii from the estimated centre of each site. Because these were highly correlated ($r = 0.98$, $P < 0.001$), 0.5 km was used.

Evaluating autofertility

Autofertility is defined as the fraction of seed production that can potentially be achieved through autonomous self-pollination, calculated by comparing fruit or seed production of flowers protected from pollinators with that of either open-pollinated or pollen-supplemented flowers (Eckert *et al.*, 2010). I used both approaches to obtain a range of autofertility values. First, in 2014, autofertility was determined in the field in a sub-set of populations (F9, HM and UB2). In each, 15 pairs of similarly sized plants were selected, for a total of 90 plants. One randomly chosen plant in each pair was enclosed in a mesh pollinator exclusion bag and the other was left open to pollinators. Up to 15 flower buds per plant were marked with non-toxic paint on the subtending pedicel (mean 9.6 ± 3.0 s.d. flower buds per plant) and fruits were collected upon ripening. Mass (mg) of each fruit was used as a surrogate for seeds per fruit, based on previous work demonstrating that fruit mass is a highly significant, linear predictor of seed mass (Spigler and Chang, 2009), which itself is a highly significant, linear predictor of seed number (Spigler and Chang, 2008). I then conducted a separate study to quantify autofertility relative to maximum seed production under controlled conditions. In Temple University's Plant Growth Facility, plants were reared from open-pollinated seeds collected from population UB5. Once plants began to flower in the pollinator-free chamber, on each of 15 plants five pairs of freshly open flowers at terminal positions were selected; one flower in each pair was designated as untreated and left unpollinated while the other was hand pollinated using outcross pollen (single donor). Fruiting success was scored for each flower, and individual fruit mass was determined.

Evaluation of PL and RA

A series of field experiments were conducted in 2014 and 2015 to test for PL and RA. In 2014, an average of 37 focal plants per population were randomly selected (± 12.1 s.d., range: 10–49, depending on population size). On each plant, after 20 % of flowers had opened on a given plant (to standardize the timing of treatments across plants), individual flowers were assigned to one of the following treatments (up to two replicates per treatment per plant): outcross pollen supplemented (S treatment) or emasculated in the bud stage (E treatment). Similar stage flowers were identified and left intact to serve as corresponding controls (C_S and C_E , respectively). Pollen for supplemental pollinations was taken from haphazardly chosen plants. Due to high rates of natural pollen removal in some populations, pollen from another population was occasionally used. Previous work has repeatedly demonstrated that crosses between *S. angularis* populations have no impact on its fruit or seed set (Dudash, 1990; Spigler *et al.*, 2017) and so should not influence the results. Focal flowers were uniquely labelled with non-toxic paint on the subtending pedicel. The date each treatment was performed was recorded and I scored whether each flower set fruit. Mature fruits were collected and individual fruit mass (mg) was determined. In 2015, I randomly selected an average of 25 plants per population (± 13.9 s.d., range: 4–49, depending on population size). The replication of treatments and corresponding controls within a plant was increased (up to five replicates) and, as a consequence, many but not all plants were used for either PL or RA, but not both. Ultimately, 15 plants per treatment per population were included on average (range: 3–25). I recorded the same data as in 2014. A total of 3101 flowers were included across years and populations.

Two important considerations should be noted. First, evaluation of PL at the plant level is considered best because it removes the potential influence of resource reallocation among flowers receiving different treatments. However, this effect was not seen in a meta-analysis when seeds per fruit was used as the variable of interest (Knight *et al.*, 2006) as is used here. Secondly, floral emasculation could negatively affect outcross pollen receipt, e.g. by reducing floral longevity, or damage flowers, causing reduced seed production and overestimation of RA (e.g. Dart and Eckert, 2013). However, emasculation does not truncate floral longevity in *S. angularis* (Spigler, 2017), and no damage to floral parts of emasculated flowers was observed. Emasculation could reduce visitation, but I do not expect this effect as only one to a few flowers were emasculated per plant. This assumption bore true in studies of a similarly pollen-rewarding Gentian, *Centaureum erythraea*, even when entire plants were emasculated (Brys *et al.*, 2011). Importantly, any unintended consequences of emasculation on visitation are distributed across all populations. To test whether emasculation negatively impacts the capacity for seed production, I added another flower treatment in a sub-set of populations in 2014 ($n = 5$ populations, mean 18 ± 2.8 s.d. plants per population): emasculated in the bud and then supplemented with outcross pollen once receptive (ES treatment). Comparison of seed production between ES and S treatments tests whether emasculation reduces the capacity for seed production.

Pollinator visitation

Pollinator observations were conducted in both years in all populations except populations F3 and PH in 2015. On a given day within each population, up to three 2×2 m plots centred on a randomly selected flowering *S. angularis* plant were observed. Within each plot, I counted flowering *S. angularis* individuals. During observation periods (mean 14 min ± 2.2 s.d., range: 10–20 min), I counted the number of visitors that entered the plot. All observations were made between 09.00 and 17.00 h. This process was repeated across multiple days. In total, a mean of 9.8 plots (± 6.4 s.d.) were observed per population, totalling 45.5 h (31 and 14.5 h in 2014 and 2015, respectively). Mean total visitation rate per plant per minute was calculated for each population as the cumulative number of visits divided by the cumulative number of *S. angularis* plants and observation minutes across plots. I divided visits into those made by putative pollinators vs. antagonists. Bees and less common flies, skippers and wasps were considered to be potential pollinators. Soft-winged beetles, however, were observed eating pollen, and are more likely to be antagonists.

Pollen deposition

In each population, stigmas were collected from up to 20 C_E flowers (mean/population: 14.3 ± 3.9 s.d., median 15) to determine pollen deposition. I collected stigmas after fruits formed, which ensured full opportunities for pollen capture over the entire floral life span and did not influence ovule fertilization. In addition, because plants were chosen randomly and plants differed in their flowering phenology (Emel *et al.*, 2017), stigmas are a random sample of pollen loads across the entire flowering season within each population. I visualized pollen grains on stigmas digested in 8 M NaOH under $\times 4$ magnification on an epifluorescence microscope and counted the number of *S. angularis* pollen grains on one of the two stigma lobes, selected at random (Spigler and Chang, 2008). I also recorded heterospecific pollen grain deposition and calculated for each population the incidence and intensity of heterospecific pollen deposition, defined as the percentage of stigmas with heterospecific pollen present and the mean percentage heterospecific pollen in the total pollen load, respectively.

Statistical analyses

Evaluating autofertility. I evaluated whether fruit set and fruit mass of bagged and open-pollinated flowers differed in the field. Fruit set was treated as a binomial response (number of fruits per number of flowers marked) and the effect of bagging evaluated using a generalized linear mixed model (proc glimmix, SAS® software Version 9.3); a general linear mixed model (proc mixed) was used to evaluate this effect on fruit mass. In both models, plant and population identity were included as random effects. For the pollinator-free growth house experiment, I only evaluated fruit mass because fruit set of focal flowers was 100 %. The effect of treatment (untreated vs. hand-pollinated) on fruit mass was evaluated using a general linear mixed model, with plant identity as a random effect. Replication of both

treatments within plants in the growth house further allowed for testing of phenotypic variation in autofertility (random treatment \times plant interaction). Autofertility was calculated as the ratio of bagged to open-pollinated fruit mass (field) or untreated to hand-pollinated fruit mass (growth house).

Testing for RA and PL. I first tested whether emasculation, *per se*, negatively impacts fruit mass. A general linear mixed model (proc mixed) was used including treatment as a fixed effect, plant and population identities as random effects and treatment date as a covariate.

To evaluate whether PL and/or RA for seeds per fruit occurred and the extent to which these depended upon population size and land use intensity, I used a general linear mixed model (proc mixed). Analysis was restricted to seeds per fruit, estimated by fruit mass, because fruit set of focal flowers was 99 %, consistent with its monocarpic life history. I included pollination treatment, population size (log-transformed), proportion of developed land, year and all two- and three-way interaction terms as fixed variables in the model (higher order interactions were not considered). Interactions were retained where significant ($P \leq 0.05$) or where they represented subsets of a significant higher order interaction. I also included treatment date of each flower, date by year interaction and the number of conspecifics within 1 m ('local neighbourhood size'), which can also influence seed production (Spigler and Chang, 2008). Plant and population identities were random effects. Sites sampled across years were treated independently. Note that I adjusted dates of E and C_E flowers to reflect the date that they were in female phase and, accordingly, to be comparable with S and C_S dates. Using the ES treatments as a reference, I determined that the median time between bud emasculation and female phase was 3 d and added this to E and C_E dates. Because I am interested in specific pairwise comparisons reflecting tests for PL (C_S vs. S) and RA (C_E vs. E) rather than omnibus tests, I used the *lsmestimate* statement in proc mixed to test *a priori* hypotheses. These tests can generate different results from Type III fixed effects for main treatment effects because, whereas Type III compares treatments setting all covariates to 0, comparisons of least squares means set covariates with their means (Littell *et al.*, 1996). The dependent variable, fruit mass, was log-transformed, which allows for inferences about differences in the ratio fruit of mass between treatments (i.e. ratios of C_S/S and C_E/E that define PL and RA, respectively) when evaluating interactions between pollination treatment and covariates. I back-transformed the least squares mean estimates of the differences between treatments, $\log S/C_S$ and $\log C_E/E$, to calculate mean PL = $1 - C_S/S$ and RA = $1 - E/C_E$, respectively. I similarly back-transformed estimates of the lower and upper bounds of the differences to determine 95 % CI for PL and RA.

To probe further the significant three-way interaction between pollination treatment, population size (log-transformed) and proportion of developed land (see the Results) and to determine whether it is specific to either PL or RA, I divided the data into two (C_S vs. S and C_E vs. E) and repeated the above analysis. As a tool to visualize the interactions, I created 3-D surfaces plotting either mean RA or PL per population as a function of population size (log-transformed) and proportion of developed land.

Evaluating population-level patterns of pollinator visitation and pollen deposition.

A general linear mixed model (proc glimmix) was used to evaluate the fixed effects of population size (log-transformed), proportion of developed land, year, visitor type (beetles vs. potential pollinators) and all two-way interactions on visitation rate. Because of the number of factors relative to the number of populations, interactions were removed from the model if they were not significant ($P > 0.05$) but main effects were retained regardless of their significance in this and all other population-level analyses. Population identity was included as a random effect to account for multiple visitor type observations per population. Visitor type was included as a grouping factor and I tested for heterogeneity of variances based on likelihood ratio scores (Littell *et al.*, 1996) because I suspected that variances were not equal among visitor types.

It was also evaluated whether *S. angularis* pollen deposition rate was predicted by visitation rate, year and their interaction. I performed three separate analyses using either total visitor, potential pollinator or beetle visitation rate as the predictor variable ($\alpha \leq 0.017$ to correct for multiple tests). Unequal variances among years was accounted and tested for based on preliminary evaluation of the data.

The influence of population size (log-transformed), proportion of developed land, year and all two-way and three-way interactions on mean *S. angularis* pollen loads and the frequency of heterospecific pollen deposition (incidence and intensity) was evaluated using general linear models (proc glm). Examination of scatterplots revealed a potentially non-monotonic relationship between conspecific pollen deposition and population size; therefore, I included a quadratic term for population size. In all analyses, residuals were checked for their conformity to model assumptions.

RESULTS

Autofertility

Sabatia angularis plants are highly capable of autonomous self-pollination. In fact, fruit set of bagged flowers (0.82 ± 0.058 s.e.) and control flowers (0.85 ± 0.06 s.e.) in the field was statistically equivalent ($F_{1,79.88} = 0.14$, $P = 0.71$), as was bagged (27 ± 2 mg s.e.) and control (29 ± 2 mg s.e.) fruit mass ($F_{1,114} = 0.66$, $P = 0.42$). Under controlled growth conditions, fruit mass for hand-pollinated flowers (63 ± 4 mg s.e.) was significantly greater than that for untreated flowers (33 ± 4 mg s.e.) ($F_{1,14,1} = 61.47$, $P < 0.001$). These translate to 93 % autofertility in the field but only 51.6 % relative to maximum seed production. The growth house study also revealed significant phenotypic variation in autofertility (i.e. significant plant \times treatment interaction: $Z = 1.99$, $P = 0.02$).

Pollination treatments

Emasculation did not damage seed set capacity of flowers. Fruit mass was equivalent between hand-pollinated, emasculated flowers and hand-pollinated, intact flowers ($F_{1,123} = 1.31$, $P = 0.25$).

Pollination treatment influenced fruit mass, but the effect varied across years and there was a significant three-way interaction between pollination treatment, population size and developed land (Table 1). Pre-planned comparisons of least squares means revealed that PL was significant on average across years, populations and all covariates ($t = 4.04$, $P < 0.0001$), and its mean was 6.5 % (Fig. 2). However, the significance of PL across years was mainly driven by the significant difference between C_S and S fruit mass in 2014 ($t = 3.93$, $P < 0.0001$), when PL was 10.4 % on average. PL was only 2.4 % on average in 2015 and not significant ($t = 1.12$, $P = 0.26$) (Fig. 2). Pre-planned comparisons further revealed an overall difference between E and C_E fruit mass across years ($t = -2.4$, $P = 0.017$), again driven by a significant difference in 2014 ($t = -2.54$, $P = 0.011$) but not 2015 ($t = -4.8$, $P = 0.63$) (Fig. 2). Thus, selfing provided RA. Its effective value, however, was relatively low: 4.0 % on average and 7.2 % in 2014 compared with only 0.6 % in 2015.

TABLE 1. Fixed effects from a general linear mixed model evaluating pollination treatment, population size, land use intensity and covariates on seeds per fruit (fruit mass)

Effect	Numerator d.f.	Denominator d.f.	<i>F</i>	<i>P</i>
Pollination treatment	3	2319	1.26	0.29
Population size (log)	1	18	8.56	0.009
Developed land (%)	1	16.3	1.75	0.20
Local neighbourhood size	1	565	5.9	0.016
Year	1	2234	61	<0.0001
Date	1	2418	365.36	<0.0001
Date × year	1	2421	67.43	<0.0001
Pollination treatment × size	3	2344	0.79	0.50
Pollination treatment × development	3	2346	3.75	0.011
Pollination treatment × year	3	2371	6.2	0.0003
Population size × land	1	16	2.37	0.14
Pollination treatment × size × development	3	2316	3.7	0.011

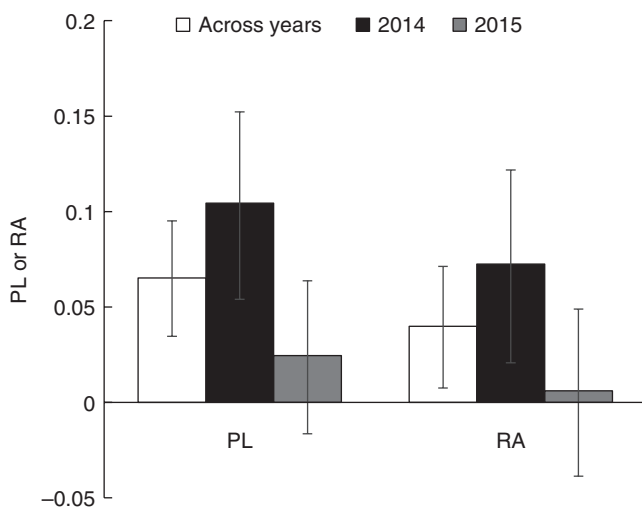


FIG. 2. Mean pollen limitation (PL) and reproductive assurance (RA) based on seeds per fruit, estimated as fruit mass (mg), on average across and within years. Back-transformed least squares means for PL and RA and their 95 % confidence interval are shown.

Evaluation of separate PL and RA models revealed that the three-way interaction between treatment, population size and development was highly significant for RA ($F_{1,876} = 10.2$, $P = 0.0015$) but not significant for PL ($F_{1,1020} = 0.14$, $P = 0.71$) (Fig. 3) (see Supplementary Data Table S2 for complete model results). Although caution must be used in extrapolating surfaces beyond the range of the data (Supplementary Data Figure S1), Fig. 3B illustrates that RA increases with declining population size under intense land use but that this effect dissipates at lower levels of development.

Additional factors influenced fruit mass (Table 1). Fruit mass increased with population size but did not vary with development on average. A significant random population effect ($Z = 2.52$, $P = 0.006$) further indicates variation

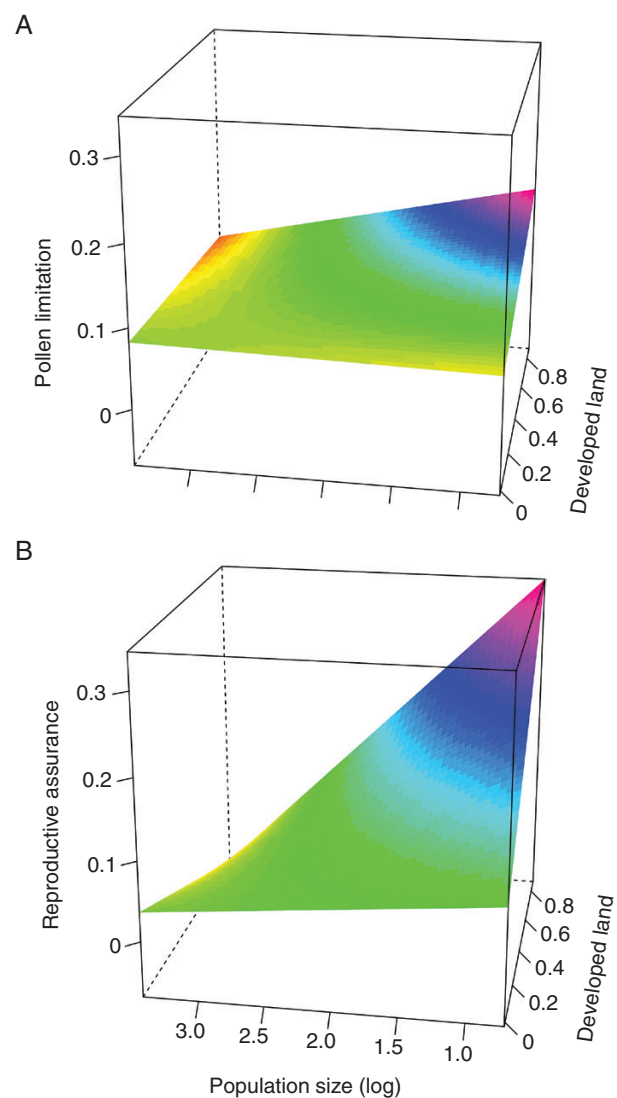


FIG. 3. Three-dimensional surface plots illustrating the interactive effects of population size (log-transformed) and proportion of developed land surrounding each population on (A) pollen limitation and (B) reproductive assurance. Note that these figures represented predicted surfaces with the reference year set to 2014. Predicted values are calculated across the entire surface (all combinations of population size and development values) based on the statistical models that use mean population parameters as the response. The distribution of data points used to create this surface is shown in Supplementary Data Fig. S1.

among populations in fruit mass beyond that explained by population size. Fruit mass was significantly higher in 2014 (34.5 ± 2.66 mg s.e.) compared with 2015 (26.3 ± 2.34 mg s.e.) and declined over the season within years, with a steeper decline in 2015 (significant date \times year interaction). In addition, local neighbourhood size had a significant negative impact on fruit mass.

Pollinator visitation and pollen receipt

In 2014, approximately half of all visits were by bees and other potential mutualist pollinators (49.4 ± 29.3 % s.d.), with the remainder representing visits by soft-winged beetles. In 2015, beetles were less abundant, representing 32.0 % of visits. Mean visitation rate significantly declined with increasing population size ($F_{1,15.9} = 9.02, P = 0.009$) but was not influenced by land use intensity ($F_{1,15.9} = 0, P = 0.95$) (Fig. 4). Mean visitation rates were similar across years ($F_{1,15.9} = 0.19, P = 0.67$) and visitor types ($F_{1,21.4} = 0.62, P = 0.44$), but variance in beetle visitation was significantly greater compared with potential pollinators ($\chi^2 = 11.84, P < 0.001$). No pairwise interactions were significant ($P > 0.25$, not shown), although power to detect significant interactions in this (and all other) population-level analysis may be limited. Sites studied in both years best illustrate the relative impacts of population size vs. land use. For example, populations of alternating generations at HM were drastically different in size (Supplementary Data Table S1), and the visitation rate of potential pollinators, in particular, was substantially lower in 2014, when the population was large, compared with 2015 when the population was small (0.22 visits per plant $\text{min}^{-1} \times 10^{-3}$ vs. 5.8 visits per plant $\text{min}^{-1} \times 10^{-3}$, respectively). In contrast, visitation rates were similar across years at sites with relatively constant population sizes. Site MB had 33

individuals in 2014 and 53 in 2015, and total visitation rates were relatively high in both years: 8.8 and 10.2 visits per plant $\text{min}^{-1} (\times 10^{-3})$, respectively. In contrast, site UB2, with similarly large populations in both years, had relatively low total visitation rates in 2014 and 2015: 1.4 vs. 2.0 visits per plant $\text{min}^{-1} (\times 10^{-3})$, respectively.

Mean conspecific pollen receipt declined with increasing total visitation rate (estimate = -4.6 ± 1.96 s.e., $F_{1,7.5} = 5.52, P = 0.049$) and varied significantly across years ($F_{1,12.91} = 22.76, P = 0.0004$). Conspecific pollen loads were nearly twice as high in 2015 than in 2014 (453.0 ± 40.9 s.e. vs. 239.9 ± 17.8 s.e., respectively) and significantly more variable in 2015 ($\chi^2 = 5.79, P = 0.02$). Analyses by visitor type revealed that the overall negative effect of total visitation is due to a strong negative impact of beetle visitation (estimate = -8.7 ± 2.63 s.e., $F_{1,7.7} = 10.87, P = 0.01$). In fact, analysis of 2014 data alone reveals that beetle visitation rate explains 58.7 % of the variation in conspecific pollen deposition among populations ($F = 9.93, P = 0.02$). Visitation by bees and other potential pollinators did not predict mean conspecific pollen deposition ($F_{1,9.4} = 0.41, P = 0.54$). The year \times visitation interaction was not significant in any of these analyses ($P > 0.40$).

Mean *S. angularis* conspecific pollen receipt, nevertheless, significantly varied with population size ($F_{1,17} = 8.11, P = 0.01$), but this relationship was non-monotonic (quadratic term: $F_{1,17} = 6.18, P = 0.02$), with intermediate-sized populations exhibiting the highest mean pollen receipt on average (Fig. 5A). Mean pollen receipt declined with increasing development, but this relationship was not statistically significant ($F_{1,17} = 3.04, P = 0.10$) (Fig. 5B). No interactions among population size, land use and year were significant ($0.13 \leq P \leq 0.93$). As in the previous analysis, year significantly influenced pollen receipt ($F_{1,17} = 18.48, P < 0.001$).

Heterospecific pollen was found on 20.1 % of stigmas examined. This frequency varied significantly among years

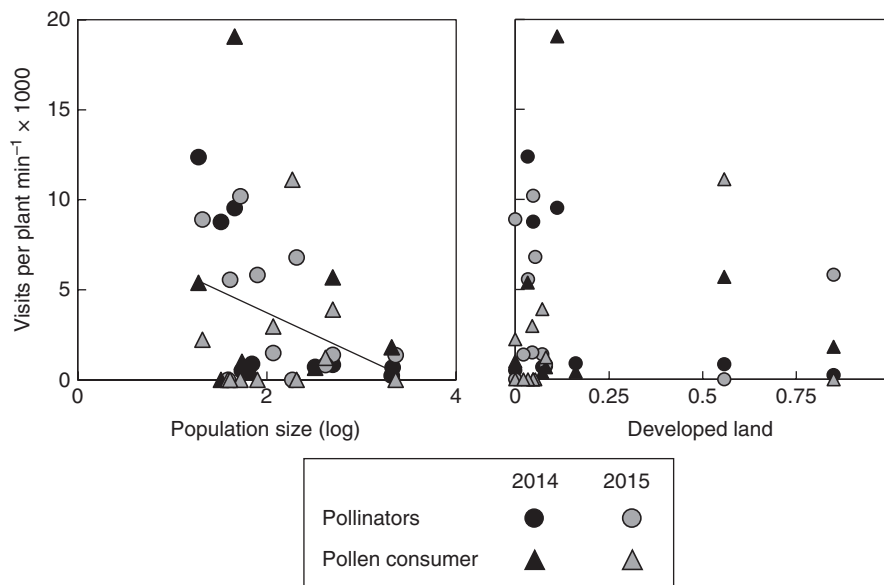


FIG. 4. Scatterplots showing the influence of population size (log-transformed) and surrounding land use intensity (proportion of developed land surrounding populations) on rates of visitation by potential pollinators vs. beetles, a potential antagonist (pollen consumer). Visitation rate is shown by year and visitor type according to the key. A regression line based on average visitation across years and pollination types represents the significant influence of population size on visitation rate.

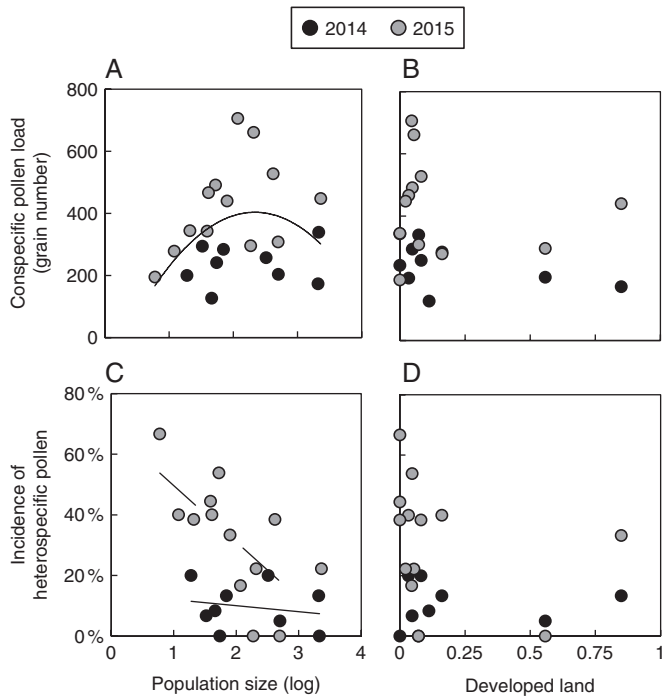


FIG. 5. Scatterplots showing the influence of population size (left panel, log-transformed) and proportion of developed land surrounding each population (right panel) on stigmatic pollen loads. Top panel: mean conspecific stigmatic pollen loads per population in relation to (A) population size and (B) proportion of developed land. Bottom panel: incidence (percentage) of stigmas with heterospecific pollen per population in relation to (C) population size and (D) proportion of developed land. Regression lines are shown where significant. Data for the 2 years are differentiated as shown in the key.

($F = 10.20$, $P = 0.005$) and was more than three times as high in 2015 than in 2014 (30.5 % vs. 9.1 %, respectively), consistent with the pattern of yearly variation in conspecific pollen loads. The percentage of flowers with heterospecific pollen within a population ranged from 0 to 67 %, and declined significantly with increasing population size ($F = 4.75$, $P = 0.04$), but not development ($F = 0.63$, $P = 0.44$) (Fig. 5C, D). The effect of population size was stronger in 2015, indicated by a significant population size \times year interaction ($F = 5.17$, $P = .04$). The final model, including population size, development, year and the size \times year interaction, explained 64 % of the variation in the incidence of heterospecific pollen deposition. The intensity of heterospecific pollen in the total pollen load was low among individuals, ranging only from 0 to 8.0 % among individuals (mean 0.30 ± 1.23 % s.d.). Mean intensity per population ranged only from 0 to 1.04 %, but similarly declined with population size ($F = 6.52$, $P = 0.02$). However, the simultaneous F -test for the final model including the main effects of population size, development and year was not significant ($F = 2.7$, $P = 0.07$).

DISCUSSION

Reproductive assurance in the most vulnerable populations may ameliorate pollen limitation

This study demonstrates that *S. angularis* is autofertile and illustrates how population size and land use can interact to

determine reliance on self-pollination in a way that is consistent with the RA hypothesis (Busch and Delph, 2012). Specifically, the highest levels of RA are predicted where *S. angularis* populations are both small and nestled within a highly developed matrix. This finding is consistent with two similar studies of the biennial *Centaureum erythraea*, which showed that RA strongly declined with population size (Brys et al., 2011) and in natural vs. highly developed areas (Brys and Jacquemyn, 2012). However, the current study is novel in revealing an interaction between population size and development. That is, for some species, small size or large habitat loss alone may minimally impact reliance on self-pollination, whereas having both leads to significant impacts. Indeed, autonomous selfing in *S. angularis* has the potential to provide between nearly complete seed production per fruit relative to pollen-limited conditions in the field and approximately half of maximum seed production. Also, while its effective value in terms of providing RA was low on average, RA reached as high as 37 % in population F3 in 2015, comprised of only six flowering plants. Even low levels of RA can impact the mating system. RA in *C. verna*, for example, which was similar to the average RA in *S. angularis* seen here, significantly increased the selfing rate (Kalisz et al., 2004). Thus, the patterns shown in this study can help explain elevated selfing rates in small, fragmented *S. angularis* populations (Spigler et al., 2010) as well as offer a mechanistic explanation for the widespread pattern of increased selfing rates seen across small, fragmented and/or disturbed populations of self-compatible species more generally (Aguilar et al., 2008; Eckert et al., 2010).

Autofertility and reliance on RA are consistent with both the low mean levels of PL seen across *S. angularis* populations and diminished variation in PL in relation to population size and development (compare Fig. 3A with B). PL was only 6.5 % on average across populations and years. This estimate is notably similar to the average 7 % PL found in another study for species with levels of autofertility on a par with *S. angularis* (González and Pérez, 2010). PL did not vary in relation to population size or land use intensity across *S. angularis* populations in this study, even in 2014 when PL was significant. In contrast, the handful of other studies that experimentally quantified PL in relation to population size or fragmentation found support for the predicted increase in PL in small or fragmented populations (reviewed in Knight et al., 2005). A key difference between *S. angularis* and focal species of those previous studies, however, is the self-compatibility of *S. angularis* and its ability to rely on autonomous self-pollination. Studies of land use impacts on PL are even rarer. Ekroos et al. (2015) similarly did not find variation in PL in relation to landscape-level metrics of land use in a study of the partially self-incompatible sticky catchfly, *Lychnis viscaria*. Interestingly, they suggested that land use might more readily impact the degree of variability in PL within populations rather than differences in mean PL.

Which mechanisms are likely to drive patterns of RA and PL?

Pollen limitation and RA are primarily predicted to arise in small populations and/or those surrounded by intense development due to reduced pollinator visitation and subsequent reductions in conspecific pollen receipt (Knight et al., 2005). Yet, in

contrast to these general expectations, visitation by potential pollinators to *S. angularis* declined with increasing *S. angularis* population size and did not vary predictably with the amount of surrounding developed land. This suggests that intra-specific competition, rather than facilitation, for pollinators occurs where *S. angularis* is more abundant, consistent with earlier work demonstrating increased PL in large experimental *S. angularis* populations (Spigler and Chang, 2009). While less common, the pattern of decreased visitation in larger plant populations has been found in a few other studies (Mustajärvi *et al.*, 2001; Campbell and Husband, 2007; Ward *et al.*, 2013). The absence of a relationship between visitation rates and surrounding land use intensity was surprising. Instead, I found low rates in more intensely developed areas, as expected, but highly variable rates in undeveloped areas. Other factors could be obscuring the impacts of development. Insects may respond instead to the proportion of surrounding agricultural land or landscape complexity *per se* (Steffan-Dewenter and Kuhn, 2003; Taki *et al.*, 2010; Ekroos *et al.*, 2013; Clough *et al.*, 2014) or to different spatial scales (Westphal *et al.*, 2006). However, effects of land use metrics including agriculture as well as consideration of larger spatial scales similarly fail to predict visitation to *S. angularis* (data not shown). The influence of population size but not landscape-scale features may instead truly reflect that at least some pollinators respond more to characteristics at the local scale (Winfree *et al.*, 2009; Williams and Winfree, 2013).

Moreover, mean pollinator visitation was a poor predictor of mean *S. angularis* pollen deposition. This disconnect between visitation rate and pollen deposition is well documented (King *et al.*, 2013). First, pollen receipt is a function of both pollinator and pollen (mate) availability. Visitation will increase pollen deposition only if pollen is available, whereas even low visitation rates could result in sufficient deposition provided pollen is abundant. Reduced mate availability in small populations combined with increased competition for pollinator visitation in the largest populations would explain the unimodal relationship between mean *S. angularis* pollen deposition and population size shown here. Secondly, not all floral visitors are efficient pollinators (Ne'eman *et al.*, 2010). Differential responses of pollinator species to fragmentation and land use can alter pollinator composition or other aspects of plant–pollinator interaction networks influencing pollination efficiency (Sjödin *et al.*, 2008; Ekroos *et al.*, 2013; Spiesman and Inouye, 2013; Theodorou *et al.*, 2017), making the composition of the visitors more important for determining pollen deposition than the visitation rate *per se*. Thirdly, I show that changes in interactions with antagonists influencing pollen deposition may also be at play. Soft-winged beetles negatively influence *S. angularis* stigmatic pollen loads. Because beetle visitation also increased with decreasing population size, their consumptive effects in small populations may have negated any benefits of increased pollinator visitation and exacerbated the issue of mate availability, contributing to PL and the need for RA. The possible impact of pollen consumption on stigmatic pollen loads and PL is also reflected in the differences in these parameters across years in the study. Stigmatic pollen loads in 2014 were nearly half of that in 2015, despite similar mean pollinator visitation rates across years. The beetle visitation rate, however, was 2-fold higher in 2014 than 2015, and in

2014 beetles represented a greater percentage of floral visitors, potentially contributing to lower conspecific pollen loads and significant PL in 2014.

Notably, mean pollen deposition did not vary predictably with land use or the interaction between land use and population size, and thus does not appear to explain variation in RA across *S. angularis* populations. Indeed, this is probably because unlike PL, RA also depends on pollen removal and self-pollen availability. Pollen deposition and removal need not be correlated (Wilson and Thomson, 1991). For example, Wilson and Thomson (1991) found that pollen deposition and receipt were decoupled in the similarly protandrous *Impatiens capensis*. In fact, among *I. capensis* patches, they found that the one with higher pollen removal had next to no pollen deposited, while another patch had relatively lower pollen removal but high pollen deposition. Such differences can have a major impact on the ability of plants to rely on selfing for RA, as low rates of pollen deposition coupled with high rates of pollen removal leave plants not only outcross pollen limited but self-pollen limited. Pollen consumption could similarly limit self-pollen availability. Consequently one hypothesis is that high levels of pollen consumption could lead to selection for greater autonomous selfing before consumers visit to provide RA (van Kleunen *et al.*, 2007). Differences in pollen removal rates among populations varying in population size and land use, perhaps due to shifts in pollinator composition, could potentially explain the pattern of RA seen across *S. angularis* populations. Future work examining changes in pollinator community and rates of pollen removal in relation to population size and land use can help inform on these dynamics.

More recently, the importance of heterospecific pollen transfer in shaping pollination dynamics has gained traction (Morales and Traveset, 2008; Ashman and Arceo-Gómez, 2013), particularly with respect to its role in reliance on autonomous self-pollination (Brys *et al.*, 2016). Heterospecific pollen transfer could be greater in small populations or those surrounded by intense development. For example, changes in pollinator foraging patterns with the relative abundance of plant species have been shown in a few studies (Kephart, 1983; Feinsinger *et al.*, 1991; Stout *et al.*, 1998), while distance to nature reserves was a critical factor determining this pollination parameter in the bee-pollinated herb *Dianella revoluta* (Duncan *et al.*, 2004). I demonstrate that the frequency of heterospecific pollen transfer in *S. angularis*, with respect to both its incidence and intensity, increased with decreasing plant population size as predicted. Although the intensity of heterospecific pollen transfer, i.e. the percentage of heterospecific pollen in the total stigmatic pollen load, is low, reaching no more than 9% on a given flower and an average of approx. 1% in any population, it is consistent with the mode across species (Ashman and Arceo-Gómez, 2013). On the other hand, this study reveals that the incidence of heterospecific pollen transfer in *S. angularis* can be high. Given its negative consequences for fruit and seed set (Morales and Traveset, 2008), the higher frequency of heterospecific pollen transfer in small populations could represent another dimension of a reproductive Allee effect, (inter)acting with reduced conspecific pollen loads to reduce mean fecundity (Waal *et al.*, 2015). However, because heterospecific pollen transfer did not vary with surrounding land use and only weakly varied with

population size in 2014, it is unlikely to explain the interactive effects of population size and land use on RA in *S. angularis*.

Finally, although not directly measured in these studies, several lines of evidence suggest that variation in resource availability could be influencing RA and PL patterns. Pollen can limit seed production only where resources are sufficient to enable plants to mature all fertilized ovules. Otherwise, where resources are limiting, PL may be relatively low or absent, even if not all ovules are fertilized (Ashman *et al.*, 2004). For example, while one might conclude that higher pollen deposition in 2015 relieved PL, resource scarcity is more likely to be responsible. Mean seeds per fruit in 2015 was actually substantially lower than in 2014, even for the supplemental pollination treatment, and the decline in seeds per fruit across the season was greater, indicating that plants were incapable of translating greater pollen loads into greater seed production in 2015. Previous work also highlights the importance of resource availability for PL in *S. angularis*, demonstrating higher PL for large plants, which presumably have greater access to resources, than for small plants (Dudash, 1993). In a similar manner, even if autonomous self-pollination increases stigmatic pollen loads, RA may be limited by resource availability. Could population variation in per capita resource availability have influenced the pattern of RA seen among *S. angularis* populations? One clue lies in the impact of local neighbourhood size on fruit mass. This and previous work demonstrate reduced seed production with increased local neighbourhood size, suggesting competition for resources (Spigler and Chang, 2008). Post-hoc analysis indicates that fruit mass is also significantly impacted by a three-way interaction between local neighborhood size, population size and land use intensity, which further suggests that the intensity of competition varies with population size and land use intensity (data not shown). Further study can explicitly evaluate how variation in per capita resource availability interacts with pollination dynamics to determine both the need and ability to rely on RA.

Conclusions

The results of this study highlight the influence of local- and landscape-level effects of changing land use on critical pollination parameters. In particular, this study demonstrates how land use intensity surrounding grassland habitats can interact with population size to influence reliance on RA and, potentially, the mating system of plant populations. Moreover, the study illustrates how patterns of variation may deviate among pollination parameters. In fact, all of the parameters measured here – pollinator visitation rates, stigmatic pollen loads, PL and RA – followed different patterns in relation to population size and land use intensity and across years, underscoring the complexity of forces in shaping these patterns. Studies that experimentally manipulate both pollen and resource availability and consider pollinator composition and pollen removal rates can help to disentangle these forces.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aoband> consist of the following. Table S1: study

site and population information. Table S2: results for fixed effects in separate PL and RA analyses. Figure S1: distribution of data points used to create the 3-D surface plots illustrating the interactive effects of population size and proportion of developed land on PL and RA. Figure S2: scatterplots showing the influence of population size and land use intensity on rates of visitation by potential pollinators vs. antagonists.

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