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Pollinator-mediated mechanisms for increased reproductive success in early flowering plants

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Mating activities change within a season in many animal and plant populations. In plants, selection towards early flowering is commonly observed. Pollinator-mediated selection is hypothesized to be a pervasive evolutionary force acting directionally on flowering time. However, pollinator-mediated mechanisms have rarely been tested in realistic field conditions, especially in perennial plants visited by a diversity of generalist insect pollinators. We examined pollinator visitation in eight *Echinacea angustifolia* populations in western Minnesota, USA, to gauge the potential for pollinator-mediated selection. *Echinacea* is a common prairie perennial that persists in isolated remnant populations. *Echinacea* is self-incompatible and is pollinated by a diversity of generalist solitary bees. A previous study found that early flowering *Echinacea* plants have higher seed set and their reproduction is less pollen-limited than late flowering plants. Twelve times throughout a flowering season, we quantified pollinator visitation rates and pollinator community composition. In three sites, we also estimated the quality of pollinator visits by examining the composition of pollinators' pollen loads brought to *Echinacea* plants. We found that three aspects of pollination dramatically decreased over the course of the flowering season. 1) Pollinators visited early flowering plants more frequently than late flowering plants. 2) The pollinator community was also less diverse late in the flowering season and became dominated by a single species of small bee, *Augochlorella aurata*. 3) Pollinators visiting *Echinacea* late in the season carried proportionally less conspecific pollen compared to pollinators visiting *Echinacea* early in the flowering season. Understanding within-season dynamics of pollination helps predict the prevalence of inbreeding, phenological assortative mating, and reproductive failure, especially in fragmented plant populations.

Keywords: plant–pollinator interactions, flowering phenology, *Echinacea angustifolia*, mate limitation, fragmentation, temporal mating patterns, pollinator-mediated mechanisms

Introduction

Mating patterns – such as who mates with whom, variation in reproductive success, and outcrossing rates – vary in both space and time (Fagan et al. 2010). For example, nearby individuals or populations exchange alleles more often than

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distant individuals or populations, and individuals with overlapping mating periods are more likely to mate than asynchronous individuals. Mating patterns that change over a season are prevalent in insects (Rowe and Ludwig 1991, Robinet et al. 2008), salmon (Morbey and Ydenberg 2003, Hendry et al. 2004), birds (Rowe et al. 1994, Kaiser et al. 2017), and are particularly common in plants (Augspurger 1981, Schmitt 1983, Kitamoto et al. 2006, Ison and Weis 2017). Mating between plants may be limited if timing of flowering among individuals does not overlap or if timing mismatches with pollinators (Augspurger 1981, Price and Waser 1998, Forrest 2015). For these reasons, among others, flowering phenology can strongly influence an individual plant's fitness. A meta-analysis of 87 species from 40 plant families found consistently higher reproductive success in early flowering individuals, especially in temperate biomes (Munguía-Rosas et al. 2011). A number of mechanisms for increased reproductive success in early flowering plants have been hypothesized that do not relate to mate limitation, including resource limitation, flowering duration, plant size, herbivory, seed predation, and seasonal constraints (reviewed by Munguía-Rosas et al. 2011, Austen et al. 2017). The extent to which the temporal dynamics of pollination service drives selection for early flowering is less understood (Elzinga et al. 2007).

Pollination service is the contribution of pollinators to plant fitness and can vary in both quantity (i.e. the number of visits or total pollen received) and quality (i.e. proportion of conspecific pollen transferred; Brown et al. 2002). For a given plant species, we can characterize three aspects of pollinators that relate to pollination service: which pollinator species are visiting (Herrera 1987, Klein et al. 2007), how many visits each taxon makes (Rush et al. 1995, Javorek et al. 2002), and the transfer of conspecific pollen during each visit (Waser 1978, Ne'eman et al. 2010). Learning about these three aspects and how they change within a season offers insight into to the extent to which pollinators may contribute to widespread variation in flowering phenology and plants fitness.

The first aspect of pollination service is the composition of the community of pollinators. It is important to note that plant–pollinator interactions are prevalent, as over 90% of flowering plants rely on insects for pollination (Buchmann and Nabhan 2012), and the majority of these are visited by multiple insect species that visit multiple plant species (Robertson 1929, Waser et al. 1996, Ollerton and Lack 1998). Just as communities of pollinators vary across space (reviewed by Williams et al. 2001), they can vary across a flowering season, leading to increased risk of extinction of flowering plants due to a demographic Allee effect (Byers and Meagher 1992, Caughley 1994, Debinski and Holt 2000). Recent studies have found high turnover in plant–pollinator interactions over the course of a season (Valverde et al. 2016, CaraDonna et al. 2017). Thus, a single assessment in time may not accurately characterize the pollinator community. If a pollinator community changes during a season, it might lead to differential pollination services over time, provided the quality of visits varies among pollinating species.

Pollination service varies among pollinator taxa for a number of reasons that are not mutually exclusive, including morphology (Forrest et al. 2011), grooming behavior (Flanagan et al. 2009, Huang et al. 2015, Parker et al. 2015), and foraging distances (Greenleaf et al. 2007, Gathmann and Tscharntke 2002). Differences between divergent taxa, such as hummingbirds versus insects, have been widely studied (Aigner 2004, Castellanos et al. 2004, Kulbaba and Worley 2013). Investigators have begun to characterize differences between solitary bee taxa, which are far more diverse and prevalent than widely studied social bees such as the honeybee *Apis mellifera* and bumble bees *Bombus* spp. (Michener 2007). Consequently, few generalizations have emerged about pollination services provided by solitary bee taxa except that their foraging ranges tend to be smaller than social bees (Gathmann and Tscharntke 2002). It remains an open question the extent to which solitary bee species differ in their contribution to pollination service of particular plant species. Given the diversity of solitary bee taxa, it seems likely that their prevalence changes over a flowering season.

The services provided by visits of a single taxon may change over time owing to nutritional needs of the foraging individuals and to the resources available. Little is known about the seasonally varying nutritional needs of solitary bees (but see Sedivy et al. 2011). But the distribution of co-flowering plant species does influence pollinators' foraging behavior and delivery of pollination services (Flanagan et al. 2011, Rafferty et al. 2013, Arceo-Gómez and Ashman 2014, Ogilvie and Thomson 2016). For example, pollinator effectiveness, calculated as total seeds per flower resulting from a pollinator visit, decreased almost 40% in a forest herb when the last plant visited was a heterospecific instead of a conspecific (Campbell and Motten 1985). Generalist pollinators often show foraging preferences towards specific plant species. Floral constancy, or fidelity, refers to when a pollinator consistently prefers one plant species to another; the less preferred species may experience reduced fitness (Campbell and Motten 1985). Pollinators often exhibit more constancy (Kunin 1997) and carry more conspecific pollen (Delmas et al. 2016) in high-density populations. Consequently, we might expect that generalist pollinators exhibit the highest fidelity to a plant species during peak flowering of that species, when conspecific plant density is highest. A reduction in floral constancy over the course of the flowering season could reduce pollination visitation rates or pollinator effectiveness due to increased interspecific pollen loss, potentially reducing reproduction among late flowering plants.

Understanding pollination services provided to plants in fragmented habitat is particularly important because many plants experience reduced reproduction due to isolation from mates, among other threats. The spatial isolation inherent in habitat fragmentation may be exacerbated, or perhaps ameliorated, by changes in the timing of mating. Variation in flowering phenology could further isolate plants from their mates and pollinators, or perhaps greater synchrony could bring them together. To investigate the dynamics of a plant-pollinator interaction over the course of a flowering season, we characterized the quality and quantity of pollinator visitation to the model prairie plant *Echinacea angustifolia* (hereafter *Echinacea*). Specifically, we quantified seasonal changes in 1) pollinator visitation rates, 2) the community of pollinators, and 3) the proportion of heterospecific grains in pollen loads on bee visitors.

Echinacea serves as an excellent system for this investigation because information exists about its pollination biology and flowering phenology and because its reproduction decreases with habitat fragmentation. *Echinacea* is a self-incompatible herbaceous perennial native to the North American plains and tallgrass prairie (Hurlburt 1999, Wagenius et al. 2010). The tallgrass prairie is one of the most threatened and least protected biomes worldwide; an estimated 1–4% of original tallgrass prairie remains (Samson and Knopf 1994, Hoekstra et al. 2005). In our study area, *Echinacea*'s pollinator community comprises over 26 species of generalist insects (Wagenius and Lyon 2010). In isolated plants, *Echinacea* reproduction is consistently pollen limited, yet many pollinators visit isolated plants (Wagenius 2006, Wagenius and Lyon 2010). In a three-year study of plants in an experimental prairie restoration, where plants were not very isolated, Ison and Wagenius (2014) observed that flowering later in the season increased pollen limitation. Learning how pollinator visitation, community composition, and pollinator foraging behavior change within a flowering season will provide insights into mechanisms of mate limitation.

Methods

Study species

Echinacea angustifolia (Asteraceae) is a long-lived (>25 years) perennial native to North American grasslands west of the Mississippi River (Wagenius et al. 2010). Upon reaching reproductive maturity, individuals may or may not flower in a given year. In flowering years, individuals typically produce one flowering head, but may produce more. Most individuals flower around 10 days but flowering varies between 6 to 36 days (Waananen et al. 2018). Each head comprises many florets (typically 80–250) which are easily visible during field surveys. *Echinacea* has a sporophytic self-incompatibility breeding system and, in our study area, reproduction is limited by receipt of compatible pollen (Wagenius et al. 2007). Previous studies indicate that annual reproductive success is limited by the availability of compatible pollen, not visitation by pollinators (Wagenius 2006, Wagenius and Lyon 2010).

Study sites and monitoring flowering phenology

Our study area is a primarily agricultural region in western Minnesota, USA (Douglas and Grant counties near 45°49´N, 95°43´W). *Echinacea* grows in small patches of remnant prairie, in isolated roadside patches, and on protected lands. We conducted this study in eight remnant *Echinacea* populations. Many of the sites were located along roadsides adjacent to agricultural fields (Table 1; Supplementary material Appendix 1 Fig. A1). The populations ranged from 21 to 215 flowering *Echinacea* plants in the summer of 2016 (27–464 flowering heads). The largest site was the east half of a 38 ha prairie reserve owned and managed by The Nature Conservancy. For this site (SPP), we used a permanent 10-meter wide transect through the site.

We characterized *Echinacea*'s flowering season by monitoring the flowering phenology of individual plants in all eight populations. We identified flowering individuals early in the season and visited each plant at least once every three days to assess flowering status. We defined an individual's flowering days as when a plant presented pollen. Due to the regular pattern of *Echinacea* floral development (Wagenius 2004), we could determine individuals' start date of flowering within one day of its occurrence. Flowering began on 18 June and ended on 17 August (Fig. 1). We used survey-grade GPS

Table 1. Characteristics of study populations. Start date is the earliest date a flowering head was observed presenting pollen at that site. End date is the last date a head was observed presenting pollen. Extent is the minimum convex polygon encompassing all flowering plants at that site in 2016. The management abbreviations are: W = managed wildlife area, R = roadside, H = undisturbed hillslope, P = managed prairie preserve.

Site	Start date	End date	Flw. plants (heads)	Extent (ha)[t]	Management
AA*	21 June	5 August	73 (80)	0.432	W/R **
EELR *	22 June	5 August	40 (52)	1.049	W
ERI	18 June	14 August	36 (46)	0.023	R
LC	21 June	31 July	99 (144)	0.430	R
LF*	21 June	28 July	153 (167)	0.427	H
RRX	24 June	29 July	48 (37)	0.172	R
STAPP	26 June	11 August	21 (27)	0.049	R
SPPE	24 June	17 August	215 (464)	3.348***	P

* Site used in pollen load analysis.

** Half of site is roadside and half is managed wildlife area on one side of road.

*** Area of the transect. The whole preserve is 38 ha.

[t] Area calculated using the R package *'adehabitatHR'* (Calenge 2006).

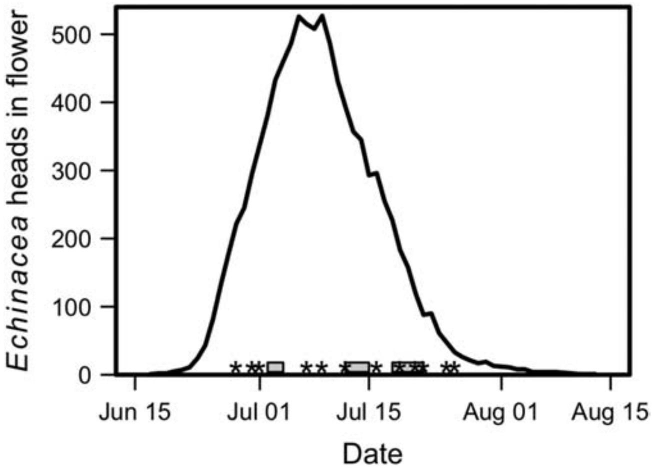

Figure 1. Total number of *Echinacea* flowering heads producing pollen per day at the eight remnant populations where we conducted pollinator observations (Table 1). The asterisks represent days when we conducted the pollinator observations. The gray bars indicate days when we collected pollinators from three of the sites (AA, EELR and LF) for the pollen load analysis.

with < 6 cm precision to map all flowering plants in all sites, except SPP where we mapped all plants in the transect and their seven nearest conspecific flowering neighbors.

Pollinator visitation and community composition

Our goal was to assess change in pollinator visitation rates and community composition throughout the flowering season. We observed sites on twelve rain-free days between 28 June and 26 July, at times of peak pollinator activity, from 8:30 a.m. to 12:30 p.m., in each of the eight *Echinacea* remnant sites (Fig. 1). On a given day, we randomly assigned eight members of our field crew (n = 12) to observe pollinator visitation of *Echinacea* in each site simultaneously. When conducting pollinator observations, we randomly selected ten plants from the site that were presenting pollen. We observed each plant for ten minutes (100 min of total observation time for each site on each observation day). When there were fewer than ten plants flowering, the researcher observed all plants in order, then repeating observations of plants in the same order until he or she had conducted ten observation periods. At two large sites (SPP and LF), populations were too large to allow efficient travel between ten randomly selected plants. To remedy this, the observer would set a transect running across the width of the site. Then, the observer would choose every second or third flowering plant to observe. We did not conduct observations at one site (RRX) during the final two days because only one plant was shedding pollen.

For each observation period, observers sat approximately one meter away from the plant oriented to avoid casting shadows over the plant. We recorded all pollinator visits during the observation period. We defined pollinators as any individuals that came into contact with the plant reproductive structures. We did not record ants or florivorous beetles on flowering heads. We used only nondestructive methods to quantify pollinator visitation and community composition to avoid depleting pollinator populations at our smallest sites. We recorded all visits using handheld video cameras or, in a few cases, an iPhone. For each pollinator visit, we recorded the plant being observed, the time of arrival, a tentative identification of the pollinator, and any distinguishing characteristics of the pollinator's appearance or behavior. We made every effort to keep the pollinator and flowering head in focus in the video.

To ensure consistent identifications, all videos were watched by three trained crew members. We identified pollinators following Droege et al. (2008) and used the reference collection of Wagenius and Lyon (2010), achieving the highest degree of taxonomic resolution possible given our non-destructive pollinator observation methods. As such, we identified some common pollinators to species and others to family or genus. Several *Lasioglossum* spp. and *Ceratina* spp. were categorized together as 'small black bees', while other *Halictus* spp. and female *Melissodes* spp. were identified as 'medium black bees'. Our coarse taxonomic categories likely lead to conservative estimates of species richness and diversity throughout the season. Videos that were out of focus or the pollinator was difficult to identify were re-watched by JLI. Occasionally (n = 9), we were unsure if a pollinator visited the focal plant multiple times within the same observation period. Our analyses yielded the same results whether potential repeat visits were counted as one or multiple visits; in our analysis, we consider all potential repeat visits as single visits. All videos are available on the Echinacea Project's YouTube Channel. Data are available from the Dryad Digital Repository (Ison et al. 2018).

Pollen loads on pollinators

To assess the composition of pollinators' pollen loads throughout *Echinacea's* flowering season, we caught pollinators early (2–4 July), mid (12–15 July), and late (18–22 July) in the flowering season (Fig. 1). During each collection period, we visited each of three sites between 7:30 a.m. and 1:00 p.m. We chose three moderately sized populations of *Echinacea*: two along roadsides (AA and EELR) and one located on an unbroken prairie hillside within a landfill facility (LF; Table 1).

We caught each pollinator in a 20 ml plastic vial as it landed on an *Echinacea* head. We caught individual bees from any species that exhibited pollinating behavior (i.e. collecting pollen or nectar or contacting plant reproductive structures). We were unable to catch any pollinating flies in the Syrphidae and did not collect florivorous beetles. We caught all pollinators within 15 s of sightings to minimize foraging on focal *Echinacea* heads. We placed pollinators in a cooler with ice packs immediately after capture to minimize grooming behavior which may lose pollen or move it from the body to scopae. In the lab, after cooling for a minimum of 30 min, we identified pollinators to genus or species and collected the pollen. We wiped bees' scopae and body with toothpicks to gather pollen. We collected scopae and body pollen separately, as they may reflect different pollen sources due to bees' foraging and grooming behavior. We did not collect scopae pollen in captured *Anthidium oblongatum* or in male *Melissodes* spp. On the same day, we released pollinators to their original site, minimizing the effect of sampling on pollinator population sizes over the flowering season.

We prepared pollen samples by wiping the pollen-coated toothpicks across 1 mm^2 fuchsin jelly melted on a glass slide (Kearns and Inouye 1993). If pollen clumped on the slide, preventing accurate pollen counts, we reheated the slide and continued spreading the jelly, or, in extremely concentrated cases ($n < 10$), we used a toothpick to remove some pollen. Once pollen was spread out, we placed a mesh of bridal veil over the jelly, acting as a grid (grid size 1 mm^2). Most slides had approximately ten grid cells with pollen in them. We did a visual sweep of the grid at a low magnification (10×) and haphazardly selected three cells containing pollen for analysis. Pollen grains were unidentifiable at this magnification, preventing bias in selection. We avoided selecting adjacent cells to account for any poor spreading of pollen. We counted the number of pollen grains of *Echinacea* and of other species in each square (magnification 400×), identifying pollen grains by comparison to a pollen catalogue created from co-flowering species at the study sites in the same summer and a pollen catalogue created previously of flowering species in our study area (<http://echinaceaproject.org>; Supplementary material Appendix 1 Table A1). To ensure consistent and unbiased identification and counts, LJP scored all of the slides in a random order and without any information about the source of the slide. In addition, we scored 10 slides a second time using the same method for selecting cells, but we did not select the same cells. The results were similar. In three slides one rare (less than five pollen grains) heterospecific taxon differed between the two counts, and in only one slide was there a common heterospecific (more than five pollen grains) that was not observed in the other count.

Pollinator visitation analysis

We tested two hypotheses related to our pollinator observations: 1) rates of pollinator visitation and 2) pollinator community composition change throughout the flowering season. We used R ver. 3.2.1 (<www.r-project.org>) and packages 'nnet' (Ripley et al. 2016), 'vegan' (Oksanen et al. 2017), and 'mateable' (Wagenius et al. 2016) for all analyses.

We evaluated the change in the rate at which pollinators visited *Echinacea* throughout the season using a generalized linear model (GLM) with site as a categorical predictor and two continuous predictor variables: date, and a weighted value of local flowering density of conspecifics surrounding each focal plant (hereafter, 'weighted local density'). Weighted local density is a weighted sum of the distance between a focal plant and its seven nearest flowering conspecifics that were flowering on the day of observation. When fewer than seven individuals were flowering within a site, we included additional flowering plants outside the focal site in calculating weighted local density. Distances were weighted using an exponential decay function such that contributions to weighted local density declined with distance (Wagenius et al. 2007, 2016; Supplementary material Appendix 1 Eq. A1). Weighted local density characterizes the local density of an individual plant on the day we observed it, thereby quantifying spatial conditions that likely affect foraging behavior of bees on that day. This measure precisely quantifies local density and is affected by a mid-season peak in flowering. Including a quadratic term of date in models would also account for effects of mid-season peak flowering on visitation, but would not be mechanistically related to bee foraging. Weighted local density and date squared are tightly correlated ($p < 0.001$; Supplementary material Appendix 1 Fig. A2), so we only included weighted local density in our model.

We conducted an initial analysis of pollinator visitation rate with the response of total pollinator visits per site per day and with a Poisson error. This model distribution yielded residual deviance greatly exceeding the residual degrees of freedom. Such overdispersion is a common problem in analyses of pollinator visit data due to frequent observations of zero pollinators (Cane 2001): we saw zero pollinators per site per day 16% of the time ($n = 15$ of 94 site–date combinations). To avoid these problems, we defined our response as the probability that at least one pollinator was observed visiting the focal *Echinacea* during a ten minute observation period as a binomial response. We added individual flowering head count as a linear predictor. To find the minimal adequate model to predict visitation probabilities, we performed backwards selection with a log-likelihood ratio selection criterion of $p = 0.05$ and beginning with a full model with all possible two-way interactions (Crawley 2012). The distribution of weighted local density among sites was uneven so we did not assess its interaction with site. Similarly, because most plants had a single flowering head (87.8% or 825 of the 940 observed plants), we did not test interactions between flowering head count and other predictors or estimate its coefficient in the model.

Pollinator community composition analysis

We assessed the pollinator community change over time using a multinomial logistic regression in the R package 'nnet' (Ripley et al. 2016) with day of year as a linear predictor. This model predicts the identity of a visitor given that a visit occurred and thus is unaffected by changes in the overall rate of pollinator visitation over time. We excluded three observations: an unidentifiable pollinator, a male *Andrena,* and a large syrphid fly. The remaining seven taxa occur between five and 121 times in the dataset. We did not model variation in the pollinator community among sites because the scarcity of data for rare species did not allow computation of standard errors for certain model coefficients. To assess changes in the diversity of pollinators, we examined Shannon diversity index (H) over time using a simple linear regression. The Shannon diversity index characterizes pollinator taxa diversity by taking into account the abundance and evenness of the pollinators. We calculated H using log base 7, scaling all values of H to between 0 and 1. Due to the high proportion of site-date combinations with visits of a single taxon (i.e. where $H = 0$), we pooled diversity across sites on each day in order to preserve normality assumptions. Because H is constrained to be non-negative, we also ran a separate non-parametric test to assess significance of the regression slope, using a permutation regression with date as predictor and H as a response using 10 000 permutations. This test found qualitatively identical results to the parametric regression. Therefore, we present results only from the parametric test.

Pollinator pollen load analysis

We tested for the effects of pollinator taxon, site, and date of capture on the proportion of total *Echinacea* pollen grains carried by pollinators and the species richness of non-*Echinacea* pollen. Because the number of the flowering plants was much lower during the third capture period, we also performed an analysis including only the first two capture periods, when the number of flowering plants was roughly the same (Fig. 1). We modeled body and scopae pollen separately to avoid pseudoreplication and because body and scopae pollen loads likely reflect different time points in a pollinator's foraging bout. We removed four bee taxa that had fewer than three samples from the analysis when testing for taxon-level differences and removed a fifth (*Lasioglossum* spp.) which was captured primarily at one site. Thus, we performed analysis with three well-represented taxa: *Agapostemon virescens, Augochlorella aurata* and *Halictus* spp. We found minimal adequate generalized linear models using a stepwise backward elimination procedure with sequential analysis of deviance and a selection criterion of $p = 0.05$ (Crawley 2012). We did not test for three-way interactions because not all factor-level combinations were represented in our dataset. Furthermore, a contingency table analysis found an uneven distribution of captured taxa at our sites ($\chi^2 = 17.304$, $df = 4$, $p < 0.01$); thus, we did not test for taxon-site interactions, as it would be difficult to discern site-taxon effects from the sampling distributions of taxa in our dataset. Thus, for all three responses (overall pollen richness and the proportion of *Echinacea* pollen on body and scopae) our full model included main effects of date, taxon, and site, as well as date–taxon and date–site interactions. We modeled proportion *Echinacea* pollen as a binomial response, using a quasibinomial error family to account for overdispersion. We modeled species richness of pollen grains using a Poisson error family. We excluded *Echinacea* pollen in richness calculations to avoid overestimating mean richness relative to variance.

Data deposition

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.t6v47p1> (Ison et al. 2018).

Results

Pollinator visitation and community composition

We observed 323 individuals from 12 taxa visiting *Echinacea* and exhibiting behavior that could lead to pollination: grooming, collecting pollen, contacting anthers or styles. After combining some taxa to make more inclusive groups (e.g. 'small black bees'), all but two observations comprised individuals from seven taxa (*Agapostemon virescens* – hereafter *Agapostemon*, *Andrena rudbeckiae* – hereafter *Andrena*, *Augochlorella aurata* – hereafter *Augochlorella,* male *Melissodes* spp., small Syrphidae flies, 'small black bees': *Ceratina* and *Lasioglossum* spp., and 'medium black bees': *Melissodes* and *Halictus* spp.). With the exception of male *Melissodes* spp., we observed all bee taxa actively collecting pollen into their scopae.

Pollinator visitation rates decreased throughout the season and the rate of decline was associated with the weighted local density of flowering *Echinacea* around the focal individual (Res. Dev. = 1057.7, $df = 920$, $p < 0.001$; Fig. 2, Table 2, Supplementary material Appendix 1 Fig. A3, Table A2). On the first day of the study the predicted probability of observing a bee visit an isolated plant (local weighted density 1.5 SD below average) within 10 min (p_{10}) was 75%, which exceeded the 48% probability of a bee visiting a plant in dense cluster (local weighted density 1.5 SD above average). This trend reversed over the course of the season, such that on the last days of observation, isolated individuals had a much lower probability of being visited (12%) than those with in clusters (29%). Investigating predicted visitation rates per site instead of for individuals based on their neighborhoods revealed decreasing pollinator visitation rates over the season at all sites except one, ERI, where the probability of visitation increased (Fig. 2B). At ERI, late-flowering individuals were in an exceptionally dense cluster of flowering plants and had many visitors. Across all sites, the mean probability of visitation in a 10 min period ranged from 24% to 92% per site on the first day of observations (28 June) and from 10% to 43% on the final day of observations (26 July). Visitation probabilities only exceeded 50% at two sites on dates after mid-season (11 July). At two sites, the visitation probability remained below 30% for the entire season.

Strong evidence supported the hypothesis that the pollinator community changed over the course of the season (Fig. 3, Supplementary material Appendix 1 Table A3). The model using date as a predictor of pollinator community composition outperformed a null model with no predictors (Dev. = 39.35, $df = 6$, $p < 0.001$). The early season

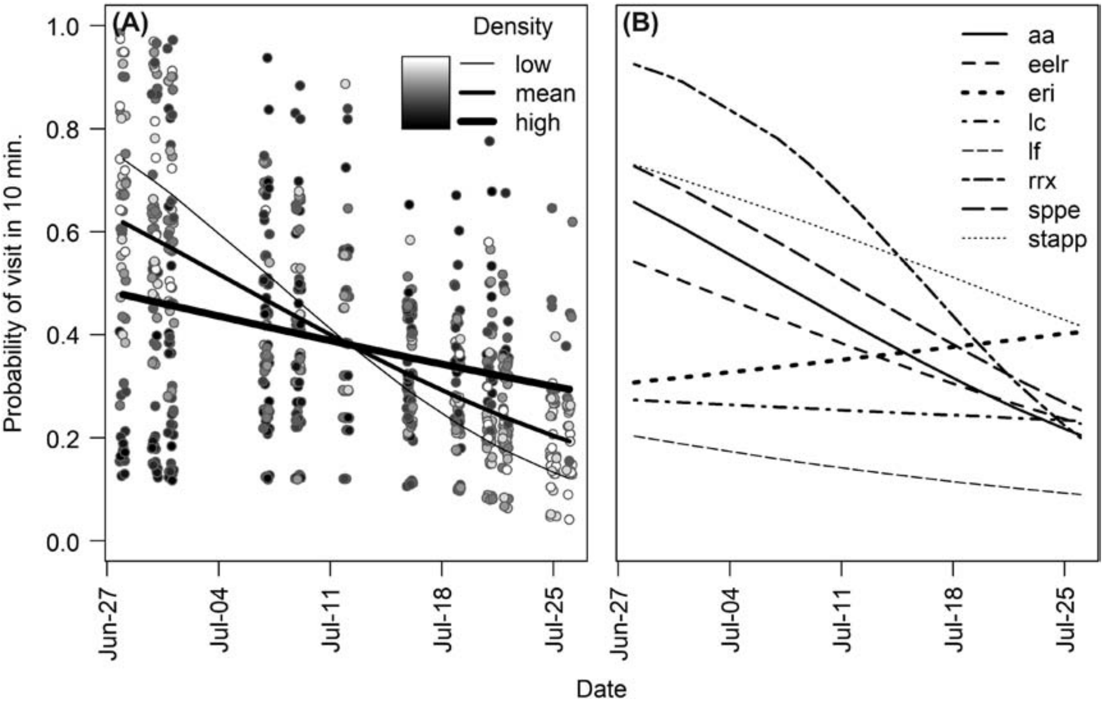

Figure 2. Visitation rates of *Echinacea* pollinators over the flowering season. Both panels show predicted values based on the minimal adequate model in Table 2 (coefficients in Supplementary material Appendix 1 Table A2). In (A), points represent predicted probability of visitation for the focal individuals on each day and shading indicates the actual weighted local density of each individual on that day. Lines in A indicate the predicted mean probability of visitation during ten minutes for individuals with high (1.5 standard deviations above the mean), mean, and low (1.5 standard deviations below the mean) weighted local density at site AA, chosen because it is a site with intermediate visitation. In (B), the lines represent the mean probability of a visit during a ten minute observation period for each of the eight remnant populations (Table 1).

(28 June – 7 July) pollinator community was composed primarily of small black bees (43% of visits on the first day), *Augochlorella* (26%) and *Agapostemon* (12%). Pollination visits by small black bees and *Agapostemon* declined to 10% and 3%, respectively over the course of the flowering season. In contrast, pollination visits by *Augochlorella* increased to 53% of all visits by the end of the season (26 July). The only other taxon making up more than 15% of the pollinator community was the small syrphid flies, which performed 18% of *Echinacea*'s total pollination visits by the end of the season (Fig. 3). Finally, diversity ranged from $H = 0.38$ to 0.77, decreasing at increments of 0.010 +/– 0.002 (1 SD), or 1%, per day (Fig. 4; $t = -4.23$, $p < 0.01$, $R^2 = 0.64$, $n = 12$).

Pollinator pollen loads

We captured and counted pollen grains from 37, 26 and 39 bees at each site (AA, EELR, LF respectively; total 102 bees, eight taxa) over the course of the season. In total, 8011 pollen grains belonging to 23 plant species (including *Echinacea*) were counted; 3604 grains (44.6%) were sampled from body slides, and 4407 (55.4%) were sampled from scopae slides. The correlation between the proportion of *Echinacea* pollen carried in body and scopae was strong ($\rho = 0.75$, $p < 0.001$).

Composition of both body and scopae pollen changed with date and rates of change differed among taxa (Fig. 5, Table 3, Supplementary material Appendix 1 Table A4). The steepest decline in proportion *Echinacea* pollen carried was

Table 2. Likelihood ratio tests for stepwise model simplification of pollinator visitation modeled as a binomial response. Deviance is the likelihood ratio test statistic. p-values are for the χ^2-test that a model simplified by excluding the focal term is not significantly different from the full model that includes the focal term. The full model included three main effects: d = day-of-year (linear predictor), w = weighted local density (linear predictor), s = site (categorical predictor, eight levels), and two interaction terms. This model is also the minimal adequate model, shown in bold. In all models, an additional term for number of flowering heads, h, was included as a linear predictor with a known coefficient of 1. Interactions are shown with ×. Parameter estimates for the minimum adequate model are shown in Fig. 2.

Model	Test Term	Res. df	Test df	Deviance	p-value
d+w+s+d×w+s×d+h		920			
d+w+s+s×d+h	d×o	921	1	5.70	0.017
d+w+s+d×w+h	s×d	927	7	19.5	0.007

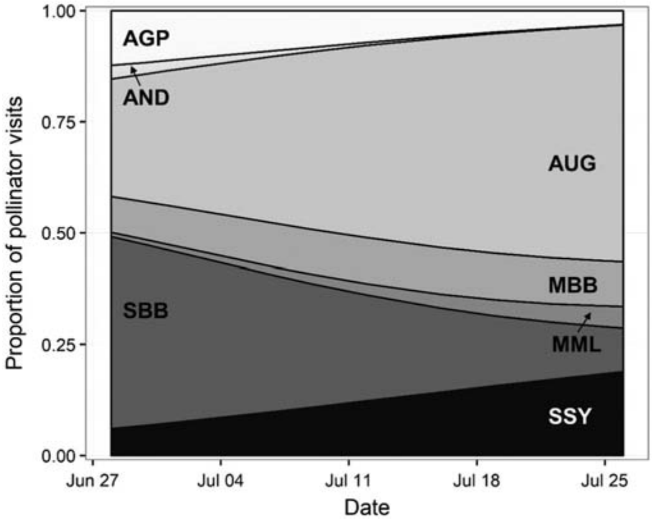

Figure 3. The composition of *Echinacea*'s pollinating community over the 2016 flowering season. The shaded areas represent the proportion of pollinator visits to *Echinacea* pooled over eight remnant sites for each pollinator taxon estimated from a multinomial logistic regression using date as a linear predictor (coefficients in Supplementary material Appendix 1 Table A3). The pollinator taxa abbreviations are as follows: AGP = *Agapostemon virescens*, AND = *Andrena rudbeckiae*, AUG =*Augochlorella aurata*, MBB = medium black bee, comprising female *Melissodes* spp. and *Halictus* spp., MML = male *Melissodes* sp., SBB = small black bee, comprising *Lasioglossum* spp., and *Ceratina* spp. and SSY = small syrphid flies.

observed in *Augochlorella*, falling from 97% on the first collection date to 18% on the final collection date for body pollen, and similarly falling from 98% to 16% in scopae pollen.

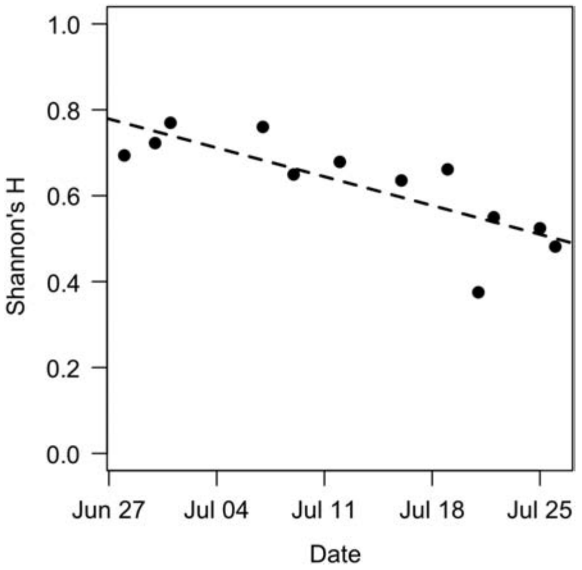

Figure 4. Diversity of *Echinacea*'s pollinating community over the flowering season. Black points are Shannon diversity index values of the pollinator community for each of 12 observation days, pooled over eight remnant sites. Black dashed line is the regression fit for observed data.

Echinacea presence in *Agapostemon* pollen loads decreased from 78% to 59% on bodies and 79% to 61% in scopae. Likewise, for *Halictus*, the decline was 87% to 68% on bodies and 88% to 77% in scopae. When we compared the first and second capture periods (when flowering plants densities were similar) we also observed a decline in proportion *Echinacea* pollen carried over time in both body ($p < 0.05$) and in scopae ($p < 0.05$; Fig. 5, Supplementary material Appendix 1 Table A5).

The mean richness of pollen carried was 2.7 species ± 1.1 (1 SE; n = 87 bees). Over 91% of these individuals were carrying four or fewer heterospecific species of pollen. We observed 3.0 ± 0.4 (1 SE) species of pollen on *Agapostemon*, 2.6 ± 0.3 (1 SE) on *Augochlorella*, and 2.5 ± 0.3 (1 SE) on *Halictus* spp. We found no significant predictors for the species richness of the pollen loads ($p > 0.05$; Supplementary material Appendix 1 Table A6).

Discussion

Seasonal patterns of reproductive success are commonly observed in flowering plants (Munguía-Rosas et al. 2011). All of the three pollinator-mediated mechanisms that we examined may contribute to seasonal declines in reproductive success: changes in pollinator visitation rate, community composition, and proportion of heterospecific grains in the pollen loads on bee visitors. We found that over the course of the season fewer pollinators visited *Echinacea;* and those that did visit carried a lower proportion of conspecific pollen (Fig. 2, 5). Meanwhile, by the end of flowering season, the composition of *Echinacea* pollinators became less diverse and dominated by one species, *Augochlorella* (Fig. 3, 4). We did not aim to quantify the relative contribution of each mechanism to overall pollination service; direct or interactive effects may predominate. However, the direction of each of the separate effects would be to reduce pollinator service over the course of *Echinacea*'s flowering season.

Temporal and spatial patterns of pollinator visitation

The present study offers insight into well-documented patterns of reproductive failure of *Echinacea* in this study system. Wagenius (2006) found that *Echinacea*'s reproduction was strongly pollen-limited and spatial isolation of indiviudal plants consistently predicted reproductive failure. Counter to expectations, Wagenius and Lyon (2010) found that pollinator visitation rates were not associated with spatial patterns of reproductive failure, in fact more isolated plants had higher rates of pollinator visitation, though, visitation rates did range widely among days. Mean daily pollinator visitation rates during 20-min observation periods for mid-sized populations or moderately isolated plants ranged between 0% and 60%. They concluded that spatial patterns of reproductive failure did not result from patterns of pollinator visitation. Further, they hypothesized that spatial patterns of reproductive failure resulted from high rates of mating

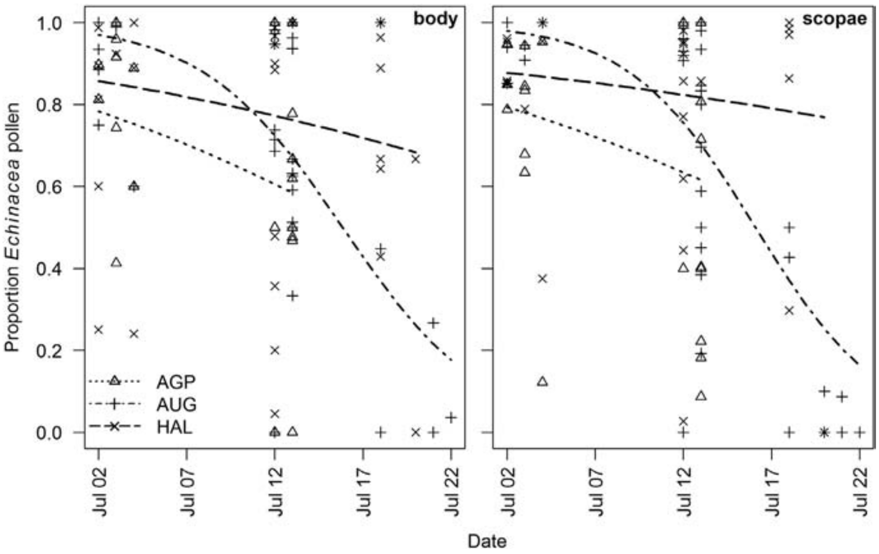

Figure 5. Proportion of *Echinacea* pollen carried on three dominant bee taxa visiting *Echinacea* throughout the flowering season. We quantified the body (left) and scopae (right) pollen loads separately for each bee. The points are the observed proportion of *Echinacea* pollen each bee carried (body n= 85, scopae n= 86). The lines represent the predicted proportion of *Echinacea* pollen based on the minimal adequate model (Table 3, coefficients in Supplementary material Appendix 1 Table A4). The pollinator taxa abbreviations are as follows: AGP = *Agapostemon virescens*, AUG = *Augochlorella aurata*, and HAL = *Halictus* spp.

incompatibility in the fragmented populations (DeMauro 1993, Wagenius et al. 2007). We can directly compare visitation results from the Wagenius and Lyon (2010) study to the current study using the transformation $p_{10} = 1 - \sqrt{(1 - p_{20})}$, where p_{10} the probability of visitation in 10 min and p_{20} is the probability of visitation in 20 min. Thus, the highest mean daily visitation rates in the previous study ($p_{20} = 0.60$) correspond to visitation of 37% in the current study. This rate is very close to the predicted mean visitation rate on July 13 (Supplementary material Appendix 1 Fig. A3). Before that date mean predicted visitation rates were higher, especially among isolated plants, and after that day mean predicted rates were lower. Other studies have found that pollinator visitation rates decrease when flowering plant densities are low (Kunin 1992, Thompson 1981). However, our results indicate that pollinator visitation rates vary over the season in a manner that cannot be explained solely by seasonal changes in flowering plant densities. Specifically, we found that isolated plants (those with low weighted local density) had above average visitation early in the season then below average later in the season (Fig. 2).

In addition to the temporal pollinator visitation patterns, we also examined variation in visitation rates among sites. Four of the eight sites had consistently low visitation throughout the season ($p_{10} < 30\%$; Fig. 2B). Similar to other studies in fragmented landscapes (Wagenius and Lyon 2010, Williams and Winfree 2013, Chen et al. 2016), our observed pollinator visitation rates appeared unrelated to the number of flowering *Echinacea* plants or size of the sites (Supplementary material Appendix 1 Fig. A1, A4). Potts et al. (2005) found that pollinator abundance may be more limited by the availability of suitable of nesting site and not floral resources.

Table 3. Likelihood ratio tests for stepwise model simplifications using backward elimination of the proportion *Echinacea* pollen observed in body and scopae pollen, model as quasibinomial response. F is the test statistic. p-values are for the *F*-test of the null hypothesis that a model simplified by excluding the focal term is not significantly different from the model on the above line that includes the focal term. The full model included three main effects: d = day-of-year (linear predictor), s = site (categorical predictor, three levels), p = pollinator taxon (categorical predictor, three levels), and two interaction terms. The minimal adequate models are indicated in bold. Parameter estimates for the minimal adequate models are shown in Fig. 5, (A) and (B), respectively.

Model: Body pollen	Test term	Res. df	Test df	F	p-value
d+p+s+d×p+d×s		75			
d+p+s+d×p	d×s	77	2	0.2555	0.78
d+p+d×p	s	79	2	0.2978	0.74
d+p	d×p	81	2	3.8951	0.02
Model: Scopae pollen					
d+p+s+d×p+d×s		76			
d+p+s+d×p	d×s	78	2	1.1286	0.33
d+p+d×p	s	80	2	3.0572	0.05
d+p	d×p	82	2	4.1874	0.02

In addition, the small solitary bees in our study likely forage short distances (150–600 m) and may not travel between sites (Gathmann and Tscharntke 2002). In our study system, Ison et al. (2014) measured pollen movement distances and found that only 2% of successful pollen movements came from a population 247 m away from the focal population, further indicating that bees are often not foraging between nearby populations.

Temporal variation in pollinator community composition and foraging behavior

In our study, *Echinacea* was visited by a diverse community of solitary generalist bees. We have clear evidence that pollination services provided by the dominant taxa consistently declined over the season and that contributions to pollination services differed among taxa. The diversity of *Echinacea*'s pollinator community decreased by more than 25% over the course of the flowering season (Fig. 3). We can interpret an *H* value of 1 as the seven pollinator taxa are equally likely to visit an *Echinacea* (i.e. zero predictability), while a value of 0 indicates only one pollinator taxon is predicted to visit an *Echinacea* (i.e. 100% predictability). Therefore, as the season progresses, *Echinacea*'s pollinator assemblage becomes 1% more predictable each day and thus less diverse. Even though all major pollinator taxa visited *Echinacea* less frequently over the course of the season, the rate of change differed among taxa. For example, we observed no *Agapostemon* visiting *Echinacea* past peak-flowering dates. *Augochlorella* also declined in its absolute visitation rate, but its presence in the dwindling assemblage of *Echinacea*'s pollinators grew larger with time, comprising more than 50% of all pollinator visits late in the season (Fig. 4).

All dominant pollinating taxa carried proportionally less *Echinacea* pollen over time, however the degree of change varied by taxon. For example, the proportion of *Echinacea* pollen carried by *Augochlorella* dropped nearly 80% across the season but dropped less than 20% in *Halictus* spp. (Fig. 4). These taxon-dependent changes in behavior underscore the importance of studying changes in the pollinator and flowering community over time. The observed decreases in *Echinacea* pollen are likely not solely a consequence of lower flowering plant densities because loads declined substantially between first capture and the second capture periods when flowering plant densities were similar (Fig. 1, 4, Supplementary material Appendix 1 Table A5).

We hypothesize that the decline in *Echinacea* pollen carried is at least in part due to a change in pollinator foraging behavior (i.e. the pollinators had decreased floral constancy). The composition of the co-flowering plant community often affects visitation rates of generalist pollinators (Flanagan et al. 2011, Arceo-Gómez and Ashman 2014). In the tallgrass prairie community, *Echinacea* tends to flower mid-season. However, there can be high year-to-year variation in the pattern and abundance of co-flowering species (Flo et al. 2018). In addition, as the flowering season progresses, the proportion of *Echinacea* heads that have finished shedding pollen increases. Pollen-foraging bees may experience increasingly negative feedback over the season as they visit more heads that have finished presenting pollen and therefore switch from *Echinacea* to co-flowering species that are still presenting pollen. Although we did not find evidence that species richness in pollen loads changed over the course of the season (Supplementary material Appendix 1 Table A4, Fig. A5), it is possible that higher-resource flowers emerged later in the season and attracted the *Echinacea*'s generalist pollinators (Supplementary material Appendix 1 Table A1).

Implications selection on phenological traits and population dynamics

The widely observed pattern that selection favors early flowering plants across species has been attributed to a number of mechanisms (e.g. resource availability, herbivory escape, competitive advantage; Munguía-Rosas et al. 2011, Austen et al. 2017). However, many of these mechanisms provide unsatisfactory explanations for the observed patterns of reproduction in *Echinacea* and potentially other pollen-limited plants (Wagenius 2006, Ison and Wagenius 2014). The changes in pollinator service that we observed across one season support the hypothesis that pollinators mediate seasonal declines in reproductive success in *Echinacea* (Ison and Wagenius 2014), which has strong implications for selection and temporal assortative mating (Weis and Kossler 2004, Ison and Weis 2017). The overall observed bee community composition, dominant taxa, and changes over time are consistent with observations in the same study area over two years (Wagenius and Lyon 2010). However, we should be careful about inferring consistency among years in other results from this one season because in the Wagenius and Lyon (2010) study the abundance of some bee taxa (small and medium black bees) did change dramatically between the two years. Furthermore, the abundance and timing of co-flowering plant species changes substantially from year to year (Ison et al. unpubl.). The timing of flowering in *Echinacea*, like in many other plant species, has a heritable component (Best and McIntyre 1972, Geber and Griffen 2003, Reed et al. unpubl.). Reed et al. (unpubl.) have documented heritable timing of flowering among *Echinacea* individuals but only after accounting for the great variation in peak days from year to year. In other words, traits for which heritability and consistency have been estimated relate to synchrony with conspecifics, but we do not know about synchrony with pollinating taxa or with co-flowering plants.

Another caveat when considering selection for flowering time is that almost all relevant investigations, including ours, (Wagenius 2006, Wagenius et al. 2007, Munguía-Rosas et al. 2011, Ison et al. 2014, Ison and Wagenius 2014, but see Austen and Weis 2016) focus exclusively on female fitness (seed set or fecundity) as a proxy for total fitness. It is plausible that effects of flowering time have different consequences for female fitness and male fitness (siring success), which makes it difficult to make inferences about the total strength and direction of selection on traits that influence flowering phenology. Nevertheless, our results indicate that variation in pollinator service within a year and among pollinating taxa is likely important for evolutionary processes, including assortative mating and perhaps selection on *Echinacea*'s flowering time, and likely other flowering plants.

Our study joins recent studies to illustrate the importance of within season temporal dynamics in plant–pollinator interactions (Valverde et al. 2016, CaraDonna et al. 2017). Our results also indicate that seasonal variation in pollination services may influence population dynamics of plants. Variation in reproductive fitness, and perhaps population growth rates, depends on synchrony with pollinators in *Echinacea* and probably many other plants with diverse solitary bee pollinators. Plants in small, isolated remnant populations suffer reduced reproduction due to spatial isolation, an Allee effect (Fagan et al. 2010). Asynchronously flowering individuals may face additional constraints to reproduction due to temporal isolation from potential mates. Such a temporal Allee effect may act independently of the spatial effect or they could be related. The strong temporal variation in pollination services within a season that we observed in this study calls into question interpretation of previous patterns of reproductive failure. Wagenius (2006) documented such failures and pollen-limitation increasing with the spatial isolation of individuals. How much of this variation in reproductive fitness could be attributed to temporal isolation? This question, though particularly relevant to *Echinacea*, warrants asking about other plants with mate-limited reproduction, especially as habitat fragmentation continues. Further efforts to characterize temporal variation in plant–pollinator interactions will increase our understanding of their ecological and evolutionary dynamics and help predict the persistence of populations in fragmented landscapes.

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Supplementary material (available online as Appendix oik-04882 at <www.oikosjournal.org/appendix/oik-04882>). Appendix 1 (Table A1–A6, Fig. A1–A5, Eq.1).