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A mechanistic model of pollinator-mediated gene flow in agricultural safflower

J.E. Cresswell*

School of Biosciences, University of Exeter, Hatherly Laboratories, Prince of Wales Road, Exeter EX4 4PS, UK

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Abstract

In many species of crop plant, gene flow by cross-pollination is possible between spatially separate fields. To preserve a crop's varietal purity or to restrict ingress into conventional varieties of genetically modified (GM) genes, a quantitative understanding of gene flow is useful. Previous measurements of gene flow in safflower (*Carthamus tinctorius* L.), a crop with GM varieties, were made in plots of less than 1 ha. Here, I evaluate a mathematical model of field-to-field gene flow due to insect pollination using parameter values appropriate to a large agricultural field of safflower. The model was solved based on laboratory pollination experiments and observations made on a large (40 ha) safflower field in Lethbridge, Canada that was pollinated by honey bees (*Apis mellifera*) and bumble bees (*Bombus* spp.). The model estimated the maximum feasible level of bee-mediated, field-to-field gene flow to range between 0.05% and 0.005% of seed set (95% upper confidence intervals of 0.23% and 0.023%), depending on the composition of the bee fauna. These relatively low values emerged for two reasons: safflower has a high capacity for automatic self-fertilization; and bees undertook long foraging bouts in the field, which made between-field pollinations relatively rare. A strategy for minimizing GM gene flow should utilize a conventional safflower variety that has a high capacity for automatic self-fertilization and should allow the plants to grow in large stands to encourage long foraging bouts by bees.

Zusammenfassung

Bei vielen Arten von Kulturpflanzen ist der Genfluss zwischen räumlich getrennten Feldern aufgrund von Kreuzbestäubung möglich. Um die Sortenreinheit einer Kulturpflanze zu erhalten oder um das Einkreuzen von Genen von genetisch modifizierten Pflanzen (GM) bei konventionellen Pflanzen zu vermeiden, ist ein quantitatives Verständnis des Genflusses hilfreich. Bisherige Untersuchungen des Genflusses bei Öldisteln (*Carthamus tinctorius* L.), einer Kulturpflanze mit GM Sorten, wurden auf Flächen kleiner als 1ha durchgeführt. An dieser Stelle evaluiere ich ein mathematisches Modell des Genflusses von Feld zu Feld aufgrund von Insektenbestäubung unter Verwendung von Parameterwerten, die für ein großes landwirtschaftliches Feld von Öldisteln wahrscheinlich sind. Das Modell basiert auf Bestäubungsexperimenten im Labor und Beobachtungen, die auf einem großen Öldistelfeld in Lethbridge, Kanada, gemacht wurden, das von Honigbienen (*Apis mellifera*) und Hummeln (Bombus spp.) bestäubt wurde. Mit dem Modell wurde der maximal mögliche Level des Genflusses von Feld zu Feld, der von Bienen vermittelt wurde, im Bereich zwischen 0,5 und 0,005% des Samenansatzes ermittelt (mit einem 95% Konfidenzintervall von 0,23 bzw. 0,023%), je

*Tel.: +44 1392 263779; fax: +44 1392 263700.
E-mail address: j.e.cresswell@ex.ac.uk

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nach dem, wie die Bienenfauna zusammengesetzt war. Diese relativ geringen Werte traten aus zwei Gründen auf: die Öldistel hat ein hohes Potenzial für eine automatische Selbstbestäubung und die Hummeln machten lange Nahrungsflüge innerhalb eines Feldes, so dass Bestäubungen zwischen den Feldern relativ selten waren. Eine Strategie, um den GM Genfluss zu minimieren, sollte eine Öldistelsorte bevorzugen, die eine hohe Kapazität für Selbstbestäubung hat, und die Pflanzen sollten in großen Beständen angebaut werden, um lange Nahrungsflüge bei den Bienen zu fördern.

Keywords: Pollination; Cross-pollination; Bumble bees; *Bombus*; Honey bees; *Apis mellifera*; *Carthamus tinctorius*; GM crops; PMF

Introduction

In an insect-pollinated crop, gene flow occurs when pollinators move between fields and cross-pollinate flowers with 'foreign' pollen. The foreign pollen on an insect body is usually exhausted at the first flowers it visits in a field (Lertzman & Gass, 1983). Thereafter, the insect cross-pollinates flowers with pollen from within the field, which reduces the relative frequency of seed set through gene flow. Below, gene flow is modelled based on the relative contribution of between-field cross-pollinations to a field's seed set (Cresswell, 2006) as follows: assume that each insect pollinator arriving at a field fertilizes b fruits, of which ψ fruits are fertilized with foreign pollen. The fraction of pollinators bringing foreign pollen is denoted by E. If the fraction of flowers pollinated by insects is denoted by R, the proportion of a field's seed resulting from insect-mediated gene flow, ξ, is

$$\xi = RE\frac{\psi}{b} \quad (1)$$

Here, empirical observations were used to evaluate the parameters of Eq. (1) appropriate to an agricultural field of safflower (*Carthamus tinctorius* L.).

Safflower is an annual, thistle-like member of the Asteraceae whose florets are borne on compact inflorescences, or capitula. In Europe and the Americas, its seeds are harvested for oil and birdseed (Dajue & Mündel, 1996). Recently, safflower has become a vehicle for plant molecular farming (PMF), which introduces GM traits for producing useful biomolecules (Horn, Woodard, & Howard, 2004). Adventitious presence of GM material with medical or industrial applications is a cause of public concern (Dale, 2005). Consequently, government regulators require knowledge to assess and manage GM confinement in PMF crops (Hill, 2005). Here, I use Eq. (1) to explore gene dispersal through insect pollination in safflower. In applying Eq. (1) to safflower, the units of ψ are capitula, rather than fruit, but the same model applies, even if multiple pollinator visits are required to fully fertilize the seed-bearing structure (Cresswell, 2003). Previous measurements of gene flow in safflower were made in plots of less than 1 ha (Deokar & Patil, 1976; McPherson, Good, Topinka, Yang, McKenzie et al., 2009). In contrast, my analysis estimates the possible levels of gene flow via insect-mediated cross-pollination into fields covering tens of hectares, which typify agricultural safflower in North America.

Material and methods

Determination of ψ

Experiments were conducted using the United States Department of Agriculture (USDA) accessions of safflower cultivar 'Royal', an agricultural variety with a high level of self-fertility (Bergman, Riveland, Flynn, Carlson, Wichman et al., 2005). I traced paternity from a homozygous dominant marker line whose flowers remained yellow on senescence (USDA accession #537601) into a homozygous recessive line whose flowers became orange on senescence (USDA accession #537600). All progeny were obtained from capitula on unmarked plants and were screened by growing to anthesis and using the colour of senescent flowers to identify the marked paternity. All plants were from a glasshouse population at the University of Exeter and only capitula in full bloom were included in the experiments, which were conducted between June 2005 and October 2006.

Paternity due to a single capitulum was quantified using the procedures of Cresswell, Osborne, and Bell (2002). Briefly, a bee visited a genetically marked capitulum and then the seed from subsequently visited unmarked capitula were screened for the marker. Visits took place in a flight cage where a genetically marked plant pruned to a single capitulum was placed in the middle of a row of unmarked plants. A bumblebee (*Bombus terrestris* L.) from a domesticated colony foraged on unmarked plants at the beginning of the row in order to become dusted with pollen and simulate pollination in mid-bout. The bee then proceeded along the line of plants, visited the marked capitulum, and subsequent visits to unmarked capitula were recorded.

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The unmarked plants were then protected from pollinators and the seed from each visited capitulum was screened for the marker.

To test for contamination of unmarked plants, individuals were randomly chosen from among those due to be exposed to bees and returned to the glasshouse, where their seed was screened for the marker. To establish that unmarked plants were capable of cross-pollination from marked plants, a capitulum from a marked plant was brushed across a capitulum on an unmarked plant randomly chosen from among those due to be exposed to bees. The unmarked plants were returned to the glasshouse, where seed from the hand-pollinated capitulum was screened for the marker.

According to Cresswell et al. (2002)

$$\psi = \sum_v v f_v \tag{2}$$

where v denotes the position of the capitulum in the bee's visit sequence after leaving the marked capitulum and f_v denotes the proportion of marked seed in the vth-visited unmarked capitulum. To quantify f_v, I fitted a decay curve to the relationship between v and the observed proportion of marked seed in the vth-visited unmarked capitulum using an exponential power function (Cresswell et al., 2002), $f_v = \exp(\alpha v^{\beta})$, where α and β are the parameters fitted to maximize a likelihood parameter, J, which quantifies the probability of occurrence of the observed number of marked seed (Eq. (3)). Let the number of seeds collected from the vth-visited unmarked capitulum be denoted by N_v, of which M_v were marked. Assume that marked seeds arise independently with a constant probability, f_v, and define J as the probability of observing M_v marked seeds among the collective N_v seeds. Let $\binom{N_v}{M_v}$ denote the number of combinations in which it is possible to allocate M_v marked seeds among N_v seeds. Then

$$J = \sum_v \binom{N_v}{M_v} f_v^{M_v} (1-f_v)^{N_v - M_v} \tag{3}$$

The estimate of ψ obtained under laboratory conditions requires adjustment, because older florets may self-fertilize and no longer be receptive to cross-pollination by the time the bee-pollination experiments took place, whereas under agricultural conditions florets are exposed to bee-pollination throughout their blooming period. I therefore increased the value of ψ by a factor of $1/r$, where r is the proportion of florets on a capitulum that were receptive during the cage experiment. To estimate r, florets opening on each of 20 plants on each day were given a day-specific paint mark. By reference to these marks, the age-specific receptivity of florets was determined by: (a) assaying the peroxidase activity of stigmatic surfaces using test papers (Dafni, Kevan, & Husband, 2005); and (b) quantifying the abundance of pollen tubes in the style under a fluorescence microscope after staining with aniline blue (Dafni et al., 2005). I then estimated r from the age distribution of florets for a capitulum in full bloom and their age-specific receptivity.

Determination of R and b

I studied pollinator behaviour in a large field of agricultural safflower (c. 40 ha; variety unknown) near Lethbridge, Canada (NeverIdle Farms Ltd.: 112° 38′ 25″ W, 49° 38′ 20″ N) between 15–17th August 2005. I recorded the density of plants and flowering capitula in ten 0.6×0.6 m quadrats located haphazardly throughout the field. The field contained both honey bees (*Apis mellifera* L.) and bumble bees (*Bombus* spp.). To estimate the density of each species, I counted the bees in 28 1.2×1.2 m quadrats at 15 m intervals along a transect through the centre of the field. To quantify pollinating activity for each bee species, I counted the number of capitula visited by bees in 1 min intervals.

I estimated the fraction of flowers pollinated by bees, R, as follows: (Cresswell, 2008). Let C denotes the density of capitula per square meter, and H denotes the time a bee takes to visit a capitulum (including inter-capitulum travel) in hours. Let B denotes the density of bees (individuals m^{-2}). If a capitulum blooms for L hours, then the number of bee visits expected by a capitulum, D, is

$$D = \frac{LB}{CH} \tag{4}$$

If $D \leq 1$ (Eq. (4)), then $R = D$, otherwise $R = 1$.

To estimate b, I followed individual bees and observed visits to successive capitula as long as possible. I noted whether each observation sequence ended because the bee left the field or because I lost sight of the bee within the field. The length of a foraging bout was then estimated from the collective observations by the number of capitula visited per departure flight.

Estimating the maximum feasible gene flow (MFG)

According to Eq. (1), the maximum feasible gene flow (hereafter 'MFG', denoted ξ_{MFG}) occurs when all bees arrive at a field bringing foreign pollen, i.e. $E = 1$, and when all flowers are visited by bees, i.e. $R = 1$. In this case, Eq. (1) simplifies to $\xi_{MFG} = \psi/b$. To estimate gene flow via two species of bee, I assume that the contribution to foreign paternity by each species is weighted according to its relative pollinating activity (Cresswell, 2006):

$$\frac{\psi}{b} = \frac{\sum_i P_i \psi_i}{\sum_i P_i b_i} \tag{5}$$

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where subscript i denotes parameters for bee species i and P_i denotes the relative proportion of pollinator visits due to bee species i. I obtained the upper confidence limit on each estimate of ψ/b by bootstrapping (Efron & Tibshirani, 1986). Specifically, the Monte Carlo methods were used to resample (with replacement) the original data that described marked inflorescences and bee residences to create a dataset with identical dimensions to the original and thereby calculating an associated value of ψ/b. From 10,000 resampling iterations, I characterized the sampling distribution of values of ψ/b, and obtained its 95th percentile. The MFG calculated using this confidence interval on ψ/b is denoted by ξ^*_{MFG}.

Results

Determination of ψ

There was no contamination of unmarked plants prior to exposure of bees (none marked among 80 progeny screened, $n=13$ plants, seed germination rate = 41%). Unmarked plants were capable of cross-fertilization by marked individuals (9% marked among 81 progeny, $n=10$ plants, germination rate = 72%). In safflower, agricultural varieties in general are capable of less than 10% outcrossing (Knowles 1969, cited in Q1 Dajue & Mündel, 1996), which suggests that variety Royal has a typically high selfing rate and that the lines used were fully able to cross-fertilize.

In cage experiments, bees visited a mean of 2.8 unmarked capitula (SE = 0.33, $n=11$) after visiting the marked capitulum. Cross-pollination by bumble bees yielded marked progeny only at the first unmarked capitulum (Table 1). The best-fit exponential power function is $f_v = \exp(-2.75v^{2.62})$ and Eq. (2) yields $\psi = 0.06$. In order to explore the statistical uncertainty of this estimate, I obtained an upper 95% confidence interval on ψ, denoted ψ_{95}, by assuming that cross-pollination yields marked progeny at only the first unmarked capitulum (see Table 1) and that binomial processes govern the proportion of first capitula that are marked (with parameter k denoting the proportion of the marked capitula) and the proportion of marked seed on each marked first capitulum (with parameter m denoting the proportion of marked seed on a marked capitulum). If there are I capitula, of which i are marked, and J seeds, of which j are marked, then probability of making these observations, P, is given by

$$P = k^i(1-k)^{I-i}\binom{I}{i} \times m^j(1-m)^{J-j}\binom{J}{j} \quad (6)$$

where $\binom{I}{i}$ denotes $I!/(I-i)!\,i!$, etc. Given the observed values of $I=8$, $i=3$, $J=24$, and $j=4$ (Table 1), an upper 95% confidence interval on the proportion of marked seed is found by maximizing the product km, subject to the constraint that $P \geq 0.05$. Using the solver subroutine in EXCEL (Microsoft Corporation, Redmond, USA), the constrained maximum is $km = 0.09$, which is also the value of ψ_{95} in the situation where a single capitulum is concerned. This result indicates that the experimental procedure for estimating ψ was fairly precise.

The peak frequency of stigmatic peroxidise activity was in two-day-old florets (proportion with detectable activity = 75%, $n=8$). The proportion of styles containing pollen tubes was 0% in one-day-old florets ($n=12$), 67% in two-day-old florets ($n=9$), and 100% in three-day-old florets ($n=12$). I therefore assume that in the absence of pollinators, florets are receptive to cross-fertilization for two days, after which automatic selfing occurs. In the laboratory, all capitula used in the bee visitation experiments had opened florets for three to four days. The proportions of florets deemed receptive (two days old or less) were 76% in capitula blooming for 3 d and 46% in 4 d, with mean = 61%, and so $r = 0.61$. Therefore, ψ adjusted for agricultural conditions is $\psi/r = 0.06/0.61 \approx 0.1$.

Table 1. Results of screening for marked progeny from unmarked capitula visited by a single bumble bee. Germination rate was 45% from total progeny of 490.

	Position in bee visit sequence					
	1	2	3	4	5	Total
Capitula screened	8	9	5	2	3	27
Progeny screened	63	73	44	10	31	221
Marked progeny	4	0	0	0	0	4
Marked capitula	3	0	0	0	0	3

Determination of R and b

In the Lethbridge field, the density of safflower was 64 individuals m^{-2} (SE = 5, $n=10$) and 88 capitula m^{-2} (SE = 5.7, $n=10$). Honey bees occurred at a density of 0.7 individuals m^{-2} (SE = 0.13, $n=28$). Bumble bees were observed foraging in the field, but none was captured in the survey. Based on the binomial theorem, the highest density of bees that could be missed by 28 quadrats with $P \geq 0.05$ is 0.07 individuals m^{-2}. Other pollinator taxa were negligibly rare. The rates at which individual bees visited capitula did not differ between bumble bees (mean = 6.8 seconds per capitulum, SE = 0.54, $n=19$) and honey bees (mean = 6.6, SE = 0.42, $n=10$).

A capitulum received a honey bee visit once every 14 min and a bumble bee visit every 148 min and therefore the expected number of visits per capitulum

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per 8 h day is $D = 38$ (Eq. (4) assuming $L = 28800$ sec, $H = 6.7$ sec, $C = 88$ capitula m^{-2}, $B = 0.77$ bees m^{-2}). As florets are receptive for two days, the proportion of florets receiving at least a single bee visit while receptive is assumed to be $R = 1$ (Eq. (4)).

I observed 1423 visits to capitula by 29 individual honey bees. Honey bees departing the field made a distinct series of grooming movements followed by a rapid vertical ascent, which coincided with the corbiculae on a bee's hind legs becoming visibly filled with pollen. Five sequences were terminated by this occurrence; otherwise, the bee was lost to view after a low-level flight across the field. Assuming that the latter flights were within-field movements, the length of a foraging bout is estimated from the number of capitula visited per departure flight, or $b_{Apis} = 1423/5 = 285$ capitula.

I observed 2389 visits to capitula by 24 individual worker bumble bees (including individuals of *B. rufocinctus* Cresson and *B. fervidus* Fabricius). I was not able to determine whether observation sequences were terminated by departure from the field, because all bumble bees were lost to view during long flights across the field and none was preceded by distinctive grooming. Therefore, I assume that all sequences were terminated by a departure flight and that, on average, a bee is initially observed halfway through its bout. Therefore, $b_{Bombus} = 2 \times 2389/24 \approx 200$ capitula.

Estimation of maximum feasible gene flow

Since honey bees and bumble bees visit safflower capitula at approximately the same rate (see results), P_i is determined by their relative abundance. Thus, $P_{Bombus} = 0.07/0.77 \approx 0.1$ and $P_{Apis} \approx 0.9$. Honey bees and bumble bees produce equivalent patterns of pollen delivery to flowers of *Brassica napus* (Cresswell, Bassom, Bell, Collins, & Kelly, 1995) and I assume the same is true in safflower; i.e. $\psi = 0.1$ for both. The maximum feasible gene flow, MFG, in safflower is then solved for various hypothetical scenarios setting $E = 1$ and $R = 1$ in Eqs. (1 and 5) and using either the various point estimates of the remaining parameters (ψ, b; solution denoted ξ_{MFG}) or the bootstrapped confidence intervals on ψ/b (solution denoted ξ^*_{MFG}) as follows:

Scenario 1: Field-to-field cross-pollination is mediated by bumble bees in a field foraged only by bumble bees. In this case

$$\xi_{MFG} = \psi/b_{Bombus} = 0.1/200 \approx 0.05\%$$
and $\xi^*_{MFG} = 0.23\%$

Scenario 2: Field-to-field cross-pollination is mediated only by bumble bees, because honey bees do not move between fields in a single foraging bout. The relative abundances of bumble bees and honey bees are as observed in Lethbridge. In this case

$$\xi_{MFG} = P_{Bombus}\ \psi/b_{Bombus} = 0.1 \times 0.1/200 \approx 0.005\% \text{ and } \xi^*_{MFG} = 0.023\%$$

Scenario 3: Field-to-field cross-pollination is mediated by bumble bees and honey bees. The relative abundances of bumble bees and honey bees are as observed in Lethbridge. In this case

$$\xi_{MFG} = \psi/[(P_{Bombus} \times b_{Bombus}) + (P_{Apis} \times b_{Apis})] = 0.1/[(0.1 \times 200) + (0.9 \times 285)] \approx 0.04\% \text{ and } \xi^*_{MFG} = 0.2\%$$

Discussion

My analysis yields point estimates of the maximum feasible level of bee-mediated gene flow (MFG) into a large agricultural field that range between 0.05% (0.23%) and 0.005% (0.023%) of seed set depending on the composition and behaviour of the pollinator fauna (95% upper confidence intervals in parentheses). MFG reaches 0.05% (0.23%) only in fields pollinated exclusively by bumble bees, but honey bees are likely to dominate the pollinator fauna of safflower in North America (Eckert, 1962; Levin & Butler, 1966). Individual honey bees often show fidelity to a particular foraging site (Beekman, 2005; Gary, Witherell, Lorenzen, & Marston, 1977) and thereby contribute little to field-to-field gene flow. In this scenario, MFG is 0.005% (0.023%). Even if pollen transfer occurs within the hive (Ramsay, Thompson, Neilson, & Mackay, 1999) to such an extent that all honey bees arrive at a field carrying pollen from another field, then MFG is 0.04% (0.2%).

The model estimates of MFG in safflower are consistent with available data, which have been collected only in experimental arrays much smaller than a typical agricultural field (McPherson et al., 2009). Accounting Q2 for patch size (Fig. 1), stands of safflower in excess of 10,000 m^2 experience incoming gene flow of less than 0.01%, which is lower than the MFG predicted by Eq. (1) for a 40 ha field under any scenario, as consistency requires.

The low values of estimated MFG have their origins in two parameters: a low value for the outcrossing parameter, ψ; and a high value for the foraging bout parameter, b. What is the basis for these values? In safflower, a low value of ψ emerged because of: (1) the high propensity for self-fertilization; and (2) the rapid attenuation of pollen carryover. The floral architecture of safflower predisposes it to self-fertilization (Howard, Howard, & Khan, 1915). As a floret opens, the stigma emerges through a tube of fused stamens and it may be covered with self-pollen before it emerges (Claasen,

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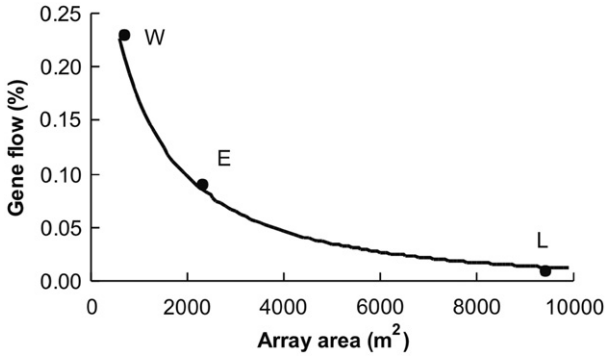

Fig. 1. Relationship between gene flow (*x*-axis; the proportion of seeds from an array of conventional safflower plants that exhibited transgenic paternity by cross-pollination from an adjacent patch of transgenic safflower plants) and the area (m^2) occupied by the conventional plants (*y*-axis). Data illustrated was reported by McPherson et al. (2009a), who studied three arrays in: Westwold, Canada (indicated by 'W'); El Bosque, Chile ('E'); and Lethbridge, Canada ('L'). Gene flow is evaluated as the mean frequency of transgenic paternity among samples taken across the entire array. Regression line: $gene\ flow = \exp(-0.1\ area^{0.413})$, $r^2 = 0.99$.

1950). In safflower, pollen carryover extends only to the next capitulum, which greatly constrains the value of ψ. By contrast, in bee-pollinated *Brassica napus*, pollen carryover from flowers preceding the last flower visited by a bee accounts for 66% of ψ , which is 1.2 fruits (Cresswell et al., 2002). Plant species with thistle-like capitula generally may be prone to low pollen carryover (Smyth & Hamrick, 1987).

The influence of within-field pollination is reflected by *b*, whose increase diminishes the level of gene flow, because inter-field cross-pollinations become rare when bees undertake long foraging bouts in a single field. Long bouts arise when single blossoms contain only a fraction of a microlitre of nectar (e.g. Zimmerman & Pyke, 1986), whereas a bee's honey sac can contain approximately 30 µl in honey bees (Ribbands, 1953) and 80 µl in bumble bees (Heinrich, 1979). Similarly, bees often visit hundreds of flowers to fill their corbiculae with pollen (Percival 1950, as cited in Ribbands, 1953). Meagre rewards in often-depleted individual blossoms probably caused the extensive foraging bouts in the safflower field studied here.

In safflower, cross-pollination creates a potential for widespread dispersal of GM pollen, because bees are capable of foraging across kilometer ranges (Cresswell, Osborne, & Goulson, 2000). What does my analysis suggest as measures to restrict the prevalence of transgenes in yields from conventional safflower? A strategy for reducing transgene ingress should minimize ψ by utilizing conventional safflower with a high capacity for selfing, and maximize *b* by growing conventional safflower in large stands. This strategy will also minimize gene flow by wind-pollination, should it occur. If honey bees contribute little to field-to-field gene flow because of strong foraging site fidelity, a further measure is to install honey bee hives near conventional fields, because the level of bumble bee-mediated gene flow declines with honey bee abundance (assuming no pollen transfer within a honey bee hive).

Two caveats are necessary. First, levels of gene flow could be higher in varieties of safflower with a greater capacity for outcrossing (Claasen, 1950). Second, where a sparse pollinator fauna fails to visit all capitula, levels of gene flow would be lower because of increased automatic self-fertilization, but bee densities would have to be reduced by almost two-orders of magnitude compared to the field studied here before each inflorescence could expect to receive less than a single bee visit (Eq. (4)).

At present, the model can estimate only the maximum feasible gene flow, because of limitations in knowledge about landscape–scale movements of bees (reflected here in setting parameter $E = 1$). Once these shortcomings are remedied, it will be possible to predict realized levels of gene flow in safflower.

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