THE ROYAL SOCIETY

The mating consequences of sexual segregation within inflorescences of flowering plants

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Many co-sexual plants segregate female and male function among flowers on an inflorescence through dichogamy or the production of unisexual flowers. Sexual segregation may reduce self-pollination among flowers within inflorescences (geitonogamy), thereby increasing the pollen available for export to other plants. To assess these complementary roles we manipulated the simultaneously hermaphroditic (adichogamous) flowers of *Eichhornia paniculata* to produce ten-flowered inflorescences with either female above male flowers (female/male inflorescences) or male/female inflorescences, which competed for mating opportunities with five-flowered adichogamous inflorescences. Because of the upward movement of bumble-bees, selfing increased upward in adichogamous inflorescences (overall female selfing rate $\bar{s} \pm$ s.e. $= 0.320 \pm 0.026$). Female flowers of male/female inflorescences selfed less than flowers in corresponding positions in adichogamous inflorescences so $\bar{s}$ fell to 0.135 ± 0.027. In contrast, all-female flowers of female/male inflorescences selfed similarly to upper flowers on adichogamous inflorescences, elevating $\bar{s}$ (0.437 ± 0.043). During 1997, male/female inflorescences sired more outcrossed seeds than female/male or adichogamous inflorescences, whereas during 1994 flowers on male/female inflorescences received fewer visits than those of adichogamous inflorescences, reducing their outcross siring success. Hence, sexual segregation limits geitonogamy and enhances outcross siring success when it does not affect pollinator behaviour, illustrating the importance of both female and male function in inflorescence design.

Keywords: dichogamy; geitonogamous self-pollination; inflorescence design; monoecy; outcross siring success; pollen discounting

1. INTRODUCTION

Hermaphroditism complicates reproduction because of the opportunity for selfing and interference between the sex roles. These consequences of hermaphroditism can be mitigated by temporally separating sex functions, so that individuals serve as only one sex at a time (Charnov 1982; Lloyd & Bawa 1984; Wasson & Newberry 1997). However, to implement this solution modular organisms with many reproductive structures, such as plants, confront the problem of segregating female and male phases of all their reproductive structures simultaneously. Alternatively, plants pollinated by vectors with stereotypic behaviour can limit sexual interference by spatially separating sex functions. For example, many bee-pollinated species with vertical inflorescences present male-phase flowers above female-phase flowers (reviewed by Bertin & Newman 1993). This pattern typically arises from the maturation of protandrous flowers from bottom to top within an inflorescence. Because bees typically forage upward on inflorescences, lower female-phase flowers should receive pollen from other plants, whereas upper male-phase flowers should disperse pollen to other plants (Darwin 1877). In addition to this structured dichogamy, angiosperms separate the sex roles spatially by other means, including monoecy (e.g. many Cucurbitaceae), andromonoecy (e.g. many Apiaceae) and gynomonoecy (e.g. many Asteraceae). For all of these forms of sexual segregation the inflorescence rather than the individual flower constitutes the fundamental unit of hermaphroditic reproduction.

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Since Darwin (1877), spatial segregation of sex functions within inflorescences has been interpreted largely as a means of reducing self-pollination and the ensuing negative consequences of self-fertilization. However, a survey by Bertin (1993) revealed that both dichogamy and monoecy occur with equal frequency among self-compatible and self-incompatible plants. Self-incompatibility effectively guards against self-fertilization, so that dichogamy and monoecy must serve as more than anti-selfing mechanisms that benefit maternal success. Lloyd & Webb (1986) suggested that these floral adaptations primarily reduced interference between female and male functions within individual flowers, thereby promoting cross-pollination (also see Bertin 1993). More recently, Harder & Barrett (1995, 1996) proposed that structured dichogamy and andromonoecy limit sexual interference between a plant's flowers. This conclusion was based on the finding that increased pollen transfer between flowers (geitonogamy) in large inflorescences reduced pollen export (pollen discounting) (Harder & Barrett 1995). Consequently, the segregation of sex functions between flowers in a manner that reduces geitonogamy should limit the loss of mating opportunities through male function for large floral displays. Unfortunately, despite widespread acceptance of Darwin's (1877) hypothesis (e.g. Proctor *et al.* 1996; Richards 1997), no empirical studies have verified that spatial segregation of sex roles provides a mating advantage relative to synchronous expression of both sex roles within flowers (adichogamy) (defined by Lloyd & Webb 1986, p. 139).

In this paper, we report on an experiment which assessed the mating success of plants with one of two

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alternate vertical arrangements of female and male flowers when they compete for mating opportunities with individuals with adichogamous flowers. Species with spatial segregation of sex roles typically present female-functioning flowers below male-functioning flowers. The opposite arrangement rarely occurs (e.g. McKone *et al.* 1995), presumably because the self-pollination that it would cause is disadvantageous (Lloyd & Webb 1986). Here we compare these two patterns of sexual segregation within inflorescences with respect to the incidence of self-fertilization resulting from geitonogamy and the associated consequences for outcrossed siring success.

To appreciate the effects of these patterns of sexual segregation on self-pollination and pollen export, consider Harder & Wilson's (1998) depiction of pollen dispersal. They proposed that two fractions of a plant's pollen contribute to the pollen on stigmas, with the remainder being left in the anthers or lost during removal from the flowers. The first fraction (a) self-pollinates in a manner which does not affect pollen export (e.g. delayed self-pollination). Pollen vectors remove a second fraction (x) which has the potential to be exported to other plants ($a + x < 1$). Before the vector leaves the plant a proportion (d) of this exportable pollen is deposited on the plant's stigmas, discounting the amount of pollen that leaves the plant. As a result, the total proportion of pollen involved in self-pollination,

$$S = a + dx, \tag{1}$$

combines the non-discounting and discounting components. Of the pollen leaving a plant, a fraction π successfully reaches stigmas, so that the proportion of pollen exported to other plants is

$$E = \pi(1 - d)x. \tag{2}$$

If the only effect of sexual segregation is to isolate the sex roles, then segregated inflorescences should differ from adichogamous inflorescences only in the proportion of exportable pollen involved in discounting (d). Based on equations (1) and (2), such variation in d alone results in a negative association between pollen export and self-pollination, so that

$$E = \pi(a + x - S). \tag{3}$$

Note that the association between a plant's male contributions to selfed and outcrossed seeds may differ from the pollination outcomes described by equation (3) because of differential success by self- and cross-pollen after pollination.

In our experiment, each trial involved arrays of equal numbers of five-flowered *Eichhornia paniculata* (Pontederiaceae) plants with adichogamous flowers and ten-flowered plants with five female and five male flowers. Both inflorescence types in an array contained the same number of functional stamens and pistils, so that our manipulations should not alter the proportion of pollen with the potential to be exported to other plants (x), but should affect the proportion of exportable pollen involved in discounting (d). Given such a situation, the outcross siring success should vary negatively with the incidence of self-pollination, as indicated by equation (3). In particular, the bumble-bees which visit these inflorescences generally move upward (see Barrett *et al.* 1994), so that inflorescences with male flowers above female flowers (male/female inflorescences) should experience less geitonogamy than either female/male or adichogamous inflorescences. To the extent that geitonogamy causes pollen discounting, we further predicted that male/female inflorescences would realize an outcross siring advantage over the competing five-flowered inflorescences, whereas female/male inflorescences would not.

2. METHODS

Eichhornia paniculata is a self-fertile, bee-pollinated species with vertical inflorescences which produce up to 20 flowers per day. The single-day, adichogamous flowers open synchronously in early morning and last for 6–8 h, depending on temperature. Although *E. paniculata* is tristylous, our experiment involved only long-styled plants, the flowers of which possess a stigma which is exerted well beyond the two anther levels, resembling a monomorphic species with approach herkogamy (stigma projecting beyond anthers) (Webb & Lloyd 1986). In *E. paniculata* mating outcomes probably reflect pollination outcomes because self and intramorph outcross pollen have equivalent pollen tube growth and siring ability (Cruzan & Barrett 1993), and fertilized seeds seldom abort (Morgan & Barrett 1989; Toppings 1989).

Each experimental array involved 18 plants with five adichogamous flowers and 18 plants with either five female above five male flowers or the opposite arrangement (one inflorescence per plant). The plants in an array all produced at least ten flowers per inflorescence, so we created inflorescences with the requisite number of flowers by removing extra flowers so as to maintain similar floral densities for both inflorescence sizes. To create female and male flowers we manipulated flowers prior to anther dehiscence. We removed either anthers or the distal portion of the style (including stigma) with fine forceps to produce female and male flowers, respectively. The two treatments involved in an array were placed in alternating positions in a 6×6 square grid (*ca.* 30 cm between adjacent plants) in a garden in Etobicoke, Ontario, Canada, during early August 1994 and 1997. After flowers closed during mid-afternoon, we marked the base of one female or adichogamous flower from each of the bottom, middle and top thirds of each inflorescence with acrylic paint. At maturity we collected the fruits produced by marked flowers, counted their seeds and sampled five seeds per fruit for electrophoretic assay. We replicated each of the two array types four times during each of the two years of data collection.

Thirty minutes after sighting the first pollinator to visit an array, we began three (1994) or four (1997) hourly, 15-min observation periods. During these observations we recorded the residence period of each bee that entered an array. We also followed a focal bee, recording the sequence of visits to inflorescences in the array and the number of flowers visited per inflorescence. During 1994, we also videotaped bees on four out of the eight arrays to quantify their movement patterns within inflorescences.

We assessed mating outcomes with respect to the competing treatments rather than individual plants using electrophoretic markers. The plants in each treatment were homozygous for a distinct *AAT-3* allele, so that the inflorescence treatment siring each seed could be identified unequivocally. In addition, we used two homozygous *PGI-2* genotypes to quantify the average proportion of seeds produced by a treatment that were self-fertilized (female selfing rate). These *PGI-2* genotypes were distributed

approximately equally among the plants of both inflorescence treatments to maximize the precision of the selfing-rate estimates. We estimated the female selfing rate and its associated standard error (based on 100 bootstrap samples) with a modification of Ritland's (1990) MLTR programme. Our enumeration of mating contributions by each treatment was based on the total seed production (N), the number of heterozygous *AAT-3* seeds (h), the number of seeds assayed electrophoretically (n) and the female selfing rate (s). The production of selfed seeds by treatment i equalled the product of its total seed production and its female selfing rate or $N_i s_i$. Correspondingly, the female production of outcrossed seeds equalled $N_i(1 - s_i)$. Finally, the total number of outcrossed seeds sired by treatment i equalled

$$N_i(1 - s_i) - \frac{h_i}{n_i}N_i + \frac{h_j}{n_j}N_j, \quad (4)$$

where the second term represents the seeds sired by treatment j on treatment i plants, and the third term represents the seeds sired by treatment i on treatment j plants.

Most of our statistical analyses employed general linear models (Neter *et al.* 1996). For analyses that involved repeated measurements of the same bee (starting position within an inflorescence, flowers visited per inflorescence and visits per flower) or array (female selfing rate and absolute siring success), we used restricted maximum likelihood to characterize the covariance between a subject's responses (Jennrich & Schluchter 1986; Proc Mixed, SAS 7.0, SAS Institute, Inc. 1998). When analysing the female selfing rate (s) we weighted the effect of each observation by the inverse of its squared standard error to account for variation in the uncertainty of estimates of s. The analysis of the number of seeds sired by a treatment in an array included two covariates. For this analysis we were specifically interested in the relation of absolute siring success to the incidence of selfing, so we included the number of selfed seeds as one covariate. To account for variation in the reproductive conditions among arrays, we included the total seed production by an array as the second covariate. The initial model for this analysis included all interactions between covariates and main effects, but we excluded non-significant interactions by backward elimination ($\alpha = 0.05$).

We used generalized linear models (McCullagh & Nelder 1989; Proc Genmod, SAS 7.0, SAS Institute, Inc. 1998) to analyse binomially distributed variables (the proportion of inflorescence visits involving segregated inflorescences, the proportion of outcrossed seeds produced in an array that were sired by segregated inflorescences and the proportion of between-flower movements that took bees up). This procedure uses likelihood ratio tests (G-tests) to identify statistically significant effects ($\alpha = 0.05$).

3. RESULTS

(a) *Pollinator behaviour*

Two bumble-bee species (*Bombus fervidus* and *Bombus vagans*) provided greater than 95% of all visits to the flowers in the arrays. The abundance of bees differed considerably between years ($F_{1,22} = 19.54$ and $p < 0.001$) with an average of 17.6 bee-minutes per 15-min observation period during 1994 (lower s.e. = 16.06 and upper s.e. = 19.20, based on square-root transformed data) compared to 29.6 bee-minutes during 1997 (lower s.e. = 27.6 and upper s.e. = 31.7).

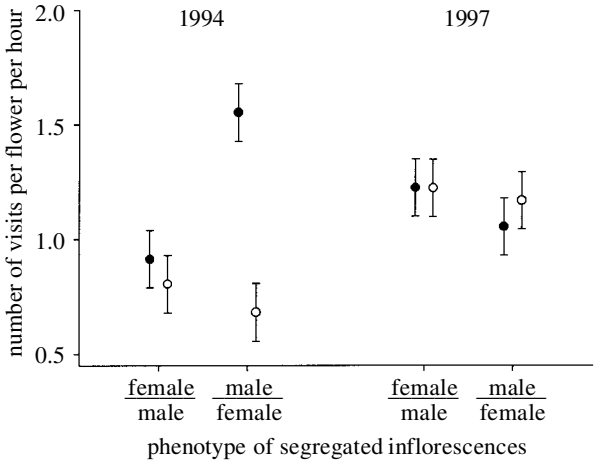

Figure 1. Mean (± s.e.) frequency of visits per flower by bumble-bees for five-flowered adichogamous (filled symbols) and ten-flowered, sexually segregated (open symbols) inflorescences of *E. paniculata*.

Based on the videotaped observations from 1994, bumble-bees generally began foraging low on inflorescences and moved upwards, as in previous array experiments (Barrett *et al.* 1994). To quantify a bee's starting position we numbered flower position sequentially from bottom to top. Based on this scale, bees began visiting five-flowered inflorescences at an average (± s.e.) position of 1.8 ± 0.16, compared to 2.7 ± 0.15 for ten-flowered inflorescences ($F_{1,297} = 17.10$ and $p < 0.001$) and this pattern did not differ between the two types of arrays ($F_{1,297} = 0.02$ and $p > 0.8$). Over 90% of movements between flowers took bees higher on inflorescences (mean = 91.1%, lower s.e. = 89.0% and upper s.e. = 92.8%) and this behaviour did not vary between array types or inflorescence types ($p > 0.4$ in all cases).

Both inflorescence size and type affected the frequency of bee visits to inflorescences and flowers within inflorescences. Bees visited ten-flowered (large) inflorescences significantly more often than five-flowered (small) inflorescences in all arrays, except those with male/female inflorescences during 1994, resulting in a significant interaction between year and array type ($G_1 = 9.55$ and $p < 0.005$). Bees also tended to visit more flowers on large inflorescences than on small inflorescences, although in arrays with male/female inflorescences they visited equal numbers of flowers per inflorescence during 1994 (year × array type × inflorescence size, $F_{1,3367} = 19.78$ and $p < 0.001$). Despite this tendency to visit more flowers on large inflorescences, the proportion visited varied negatively with flower number, counteracting bees' general preferences for large inflorescences, so that flowers on small and large inflorescences received visits at the same rate (figure 1). The only exception to this equal visitation involved male/female inflorescences during 1994, which received fewer visits per flower per hour than the competing adichogamous inflorescences. This inconsistency caused an interaction between year, array type and inflorescence size ($F_{1,24} = 5.15$ and $p < 0.05$).

(b) *Female selfing*

The relation of the female selfing rate to flower position within inflorescences is completely consistent with

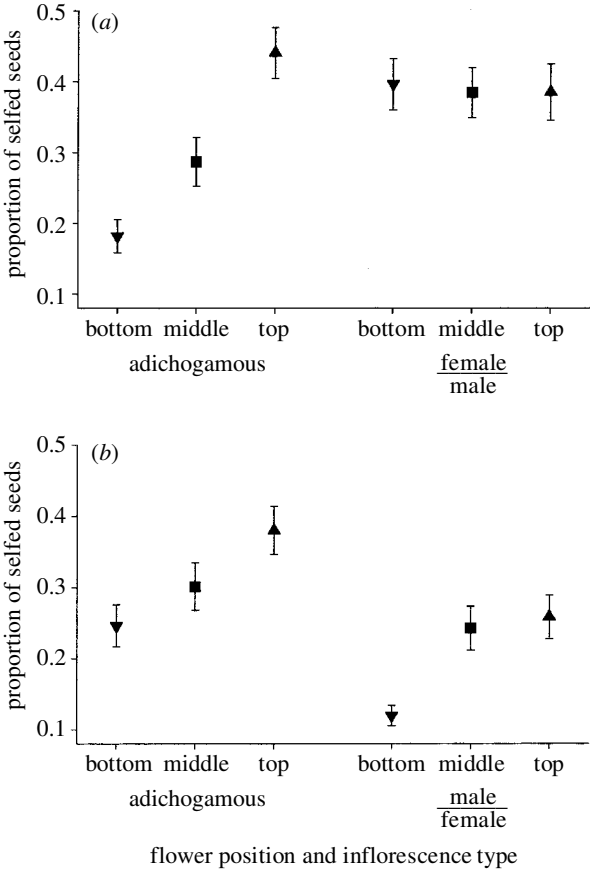

Figure 2. Mean (± s.e.) female selfing rates for flowers at different positions within adichogamous and sexually segregated inflorescences of *E. paniculata*. (*a*) Arrays containing female/male inflorescences and (*b*) those with male/female inflorescences.

geitonogamous self-pollination by upward foraging bees (figure 2). Within inflorescences with adichogamous flowers the fraction of selfed seeds approximately doubled between bottom and top flowers, reaching a maximum of around 0.4. Placement of female flowers above male flowers eliminated this increasing pattern and resulted in female flowers at all positions experiencing equivalent selfing to upper flowers on adichogamous inflorescences (figure 2*a*). The contrasting arrangement of male flowers above female flowers reduced the overall female selfing rate, with bottom female flowers experiencing half as much selfing as bottom flowers on adichogamous inflorescences (figure 2*b*). These contrasting patterns resulted in a significant interaction between array type, flower type and flower position ($F_{2,72}=5.36$ and $p<0.01$), which did not differ between years ($F_{2,72}=1.68$ and $p>0.1$), even though the overall selfing rates were slightly higher during 1997 ($\bar{s}\pm$s.e. $=0.32\pm0.013$) than 1994 (0.28 ± 0.013) (year effect, $F_{1,72}=5.14$ and $p<0.05$).

(c) *Outcross siring success*

In contrast to female selfing, the outcross siring success of sexually segregated inflorescences differed between years (figures 3 and 4). During 1997, when flowers on competing inflorescence types experienced equal visitation rates, the number of seeds sired by outcrossing declined with increases in the number of selfed seeds for a given inflorescence type (partial regression coefficient

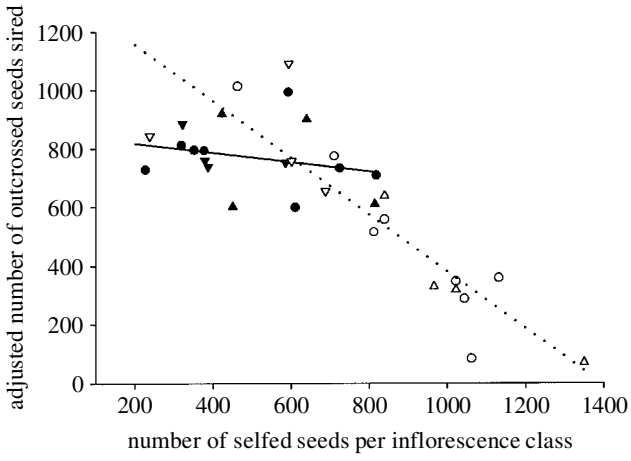

Figure 3. Relation of outcross siring success to selfed seed production during 1994 (filled symbols and solid line) and 1997 (open symbols and dotted line) in experimental arrays of *E. paniculata*. Circles indicate adichogamous inflorescences, triangles represent female/male inflorescences and inverted triangles depict male/female inflorescences. The number of outcrossed seeds has been adjusted to account for the variation between arrays in total seed production.

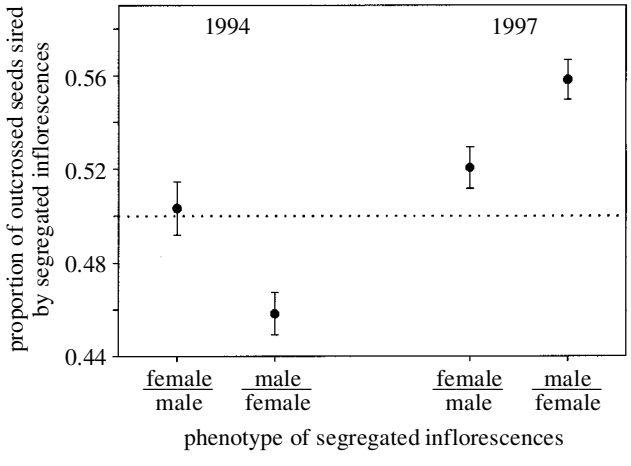

Figure 4. Mean (± 95% confidence interval) relative outcross siring advantage of sexually segregated inflorescences over adichogamous inflorescences of *E. paniculata*. The dashed line represents equal outcross siring success.

$\pm$ s.e. $=-0.969\pm0.217$, $t_{21}=4.46$ and $p<0.001$; figure 3). In contrast, during 1994 outcrossed siring success did not vary significantly with selfed seed production (-0.160 ± 0.262, $t_{21}=0.61$ and $p>0.5$), resulting in a significant interaction between the effects of year and selfed seed production ($F_{1,21}=7.11$, $p<0.025$).

The outcross siring success of the two types of sexually segregated inflorescences relative to adichogamous inflorescences also differed between years (year × array type interaction, $G_1=73.05$ and $p<0.001$; figure 4). Female/male inflorescences sired more outcrossed seed than adichogamous inflorescences during 1997, but realized no advantage during 1994. Not surprisingly, given the relatively infrequent visits to flowers on male/female inflorescences during 1994 (figure 1), they suffered a significant siring disadvantage. However, during 1997 male/female inflorescences sired 56% of the outcrossed seeds, compared to the 44% sired by adichogamous inflorescences.

4. DISCUSSION

As Darwin (1877) originally proposed, the vertical segregation of sex function in bee-pollinated plants strongly determines the incidence of geitonogamous selfing (figure 2). Relative to adichogamous inflorescences, the placement of male flowers above female flowers greatly reduces selfing at corresponding positions within the inflorescence and for the inflorescence as a whole. This diminished selfing probably reflects both the elimination of intrafloral selfing resulting from sexual segregation and a reduction in geitonogamy. In contrast, when female flowers occur above male flowers they all receive self-pollen as though they were upper flowers in adichogamous inflorescences, greatly elevating the plant's overall female selfing rate. Therefore, given strong inbreeding depression, female/male plants would realize lower female success than adichogamous plants, which in turn would be less successful than male/female plants. However, with weak inbreeding depression other mechanisms are necessary to explain the rarity of the female/male condition among bee-pollinated plants.

In addition to providing the first experimental evidence supporting Darwin's (1877) hypothesis on the effects of male/female inflorescences on self-pollination, our 1997 results demonstrate the benefits of this pattern of sex segregation for male outcrossing. During 1997, male/female inflorescences sired more seeds than either adichogamous or female/male inflorescences (figure 4). This outcome reflects less pollen discounting by male/female inflorescences during a year when each additional selfed seed diminished the outcrossed siring success by one seed (figure 3). As Lloyd (1988, 1992) proposed, every pollen grain deposited geitonogamously could be exported to other plants, thereby causing a one-to-one loss of outcross siring opportunities.

Our results also reveal several outcomes not expected from our initial portrayal of the influence of sexual segregation on pollination, but which can be understood in the context of the diversity of pollen fates and their interactions. First, the outcross siring success of female/male inflorescences equalled (1994) or exceeded (1997) that of competing adichogamous inflorescences (figure 4). This result arose even though the incidence of selfing within female/male inflorescences indicates intensified geitonogamy, which should have exacerbated pollen discounting. On a sexually segregated inflorescence, geitonogamy only occurs when pollinators visit male and then female flowers. When this sequence is violated, the separation of sex roles will limit pollen discounting relative to adichogamous inflorescences, regardless of the inflorescence architecture. Out of the 61 videotaped observations of female/male inflorescences during which a bee visited at least one male flower, 13% involved visits to male flowers only. This behaviour would cause no discounting, thereby elevating the siring success relative to adichogamous inflorescences.

The second unexpected result was that male/female inflorescences sired fewer outcross seeds than adichogamous inflorescences during 1994 (figure 4), even though these inflorescences exhibited the same pattern of selfing observed during 1997. During the 1994 trials, bees visited flowers on male/female inflorescences half as often as those on adichogamous inflorescences (figure 1). Although the reasons for this behaviour remain unexplained, it seems likely that bees removed less pollen from male/female inflorescences than from adichogamous inflorescences. Such a reduction violates our assumption that the competing inflorescence types contributed equally to the population of pollen with the potential to be exported to other plants (i.e. x is equal for both inflorescence types) which underlies our prediction of a negative association between pollen export and self-pollination (equation (3)). This decrease in potentially exportable pollen counteracts the reduction of geitonogamous pollen discounting (i.e. smaller d) associated with the male/female arrangement (figure 2*b*). Indeed, adichogamous inflorescences (A) export more pollen than dichogamous inflorescences (D) when their relative advantage through the removal of potentially exportable pollen exceeds their relative disadvantage through pollen discounting,

$$\frac{x_{\mathrm{A}}}{x_{\mathrm{D}}} > \frac{1 - d_{\mathrm{D}}}{1 - d_{\mathrm{A}}}. \tag{5}$$

This pollen-export advantage occurs despite greater pollen discounting by adichogamous inflorescences. This conclusion illustrates that lack of a negative relation between outcross siring success and selfing (figure 3) by itself provides limited insight into the occurrence or intensity of pollen discounting and its impact on plant mating (also see Harder & Wilson 1998; Harder 2000). Furthermore, the results from 1994 remind us that sexual segregation can affect aspects of pollination other than pollen discounting and that the evolution of segregation depends on its cumulative mating consequences.

Structured dichogamy and monoecy have been affiliated as mechanisms which limit selfing (reviewed by Bertin 1993); however, these forms of sexual segregation include species with fundamentally different pollination systems. Species with structured dichogamy are generally pollinated by pollinators with stereotypic behaviour (Bertin & Newman 1993) which can be manipulated by inflorescence architecture. Many of these species present female- and male-phase flowers simultaneously, so that spatial separation alone limits geitonogamy and its consequences. In contrast, many monoecious species are pollinated either by pollinators with less predictable behaviour or abiotically (Bawa & Beach 1981; Webb & Lloyd 1986). Presumably because such uncertain vector behaviour confounds the anti-geitonogamy role of spatial segregation, monoecious species also typically present female and male flowers at different times. Our experiment simulated the first situation and so may identify the major influences on the evolution of structured dichogamy. In contrast, the specific pollination consequences of sexual segregation that we observed may be less important in the evolution of monoecy, which probably depends on the additional benefits of segregation, such as flexibility for altering female and male investment (Bawa & Beach 1981; Willson 1983).

Our experiment equalized the female and male reproductive potentials between treatments, but in doing so it created an atypical pattern of sexual segregation for species with structured dichogamy. In such species the female and male phases of individual flowers commonly

differ in duration (reviewed by Bell & Cresswell 1998), causing corresponding inequality in the numbers of female- and male-phase flowers. In addition, the sex phases often differ in their availability of rewards for pollinators (reviewed by Aizen & Basilio 1998). To date, such differences have been interpreted as adaptations which accommodate different rates of increase in female and male success by individual flowers with successive pollinator visits (Lloyd & Yates 1982; Harder & Thomson 1989; Brunet & Charlesworth 1995; also see Bell & Cresswell 1998). However, differences in the number and nectar content of female- and male-phase flowers could also affect geitonogamy and its associated pollen discounting by manipulating pollinator behaviour. Hence, the most effective implementation of structured dichogamy may often require more than the simple segregation of sex roles within an inflorescence. Furthermore, the evolution of floral characteristics in these species probably depends on their consequences for the operation of the inflorescence as a whole. Such considerations imply that many correlations between floral and inflorescence traits in species with structured dichogamy await exploration.

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