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THE EVOLUTION OF DISTYLY: POLLEN TRANSFER IN ARTIFICIAL FLOWERS

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Abstract.—A recent model by Lloyd and Webb derives conditions necessary for the evolution of distyly based on pollen-transfer probabilities between ancestral morphs and invading mutants. We used bumblebees visiting artificial flowers to measure the parameters of the model. Our findings supported the first evolutionary step proposed by the model, establishment of a stigma-height polymorphism. Conditions for the subsequent establishment of an anther-height polymorphism were not satisfied by pollen-transfer patterns alone. Because conditions for the first step are considered more onerous, however, and because the second stage depends on inbreeding depression as well as pollen-transfer patterns, we interpret our results as supporting the plausibility of the Lloyd-Webb model. Video images of bees visiting glass-sided artificial flowers demonstrate a mechanism for disassortative pollination between the ancestral and mutant morph. In general, pollen-transfer probabilities were negatively correlated with the height difference between anthers of the donor and stigma of the recipient. Style length affects bee feeding posture in such a way that disassortative pollination could feasibly occur in the absence of an anther-height polymorphism.

Key words.—Artificial flowers, *Bombus,* distyly, evolution, herkogamy, heterostyly, pollination.

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Distyly is a balanced polymorphism in which about half of the plants in a population have long styles and short stamens, whereas the remainder possess short styles and long stamens. It has evolved independently at least 23 times (Ganders 1979; Lloyd and Webb 1992a). Since Darwin's early (1877) work on distyly, two quantitative models have been put forward suggesting possible stages in its evolution (Charlesworth and Charlesworth 1979; Lloyd and Webb 1992b). Such models must explain two evolutionary events: the development of the two morphological groups, and the dimorphic incompatibility system characteristic of distylous plants, whereby only crosses between different morphs are fertile.

The Charlesworths' model (1979) showed that any change in stigma height is unlikely to become established as a polymorphism in a self-compatible species with anthers and stigma originally borne at the same height. Any change that reduced pollen receipt would be lost, whereas a change that increased receipt would sweep through the population, preventing the establishment of a polymorphism. According to this view, the necessary first stage is the development of dimorphic self-incompatibility, which could become established if inbreeding depression and selfing rates are high enough to favor the development of a morph with sterile pollen. A linked mutation for a new stigma type compatible with the new pollen type would complete the establishment of dimorphic self-incompatibility. Later, the morphological groups could evolve, if distyly increased disassortative mating between the two mating groups.

Most workers have accepted the sequence outlined above, at least the proposition that morphological groups evolved following the establishment of incompatibility groups. An important variation on the assumptions of the model was suggested by Ganders (1979). He proposed that the ancestral plants may have been herkogamous, that is, the anthers and stigma were spatially separated within flowers. He argues that this morphology is more often associated with outcrossing plants such as the putative ancestors of distylous taxa.

In a fundamentally different scenario, Lloyd and Webb (1992b) proposed that distyly could have evolved through two morphological changes preceding the establishment of the incompatibility groups (reviewed by Barrett 1990). As in Ganders (1979), the ancestor is assumed to be herkogamous. Specifically, the ancestor is postulated to have "approach herkogamy," in which the stigma is borne at a higher level than the anthers (Webb and Lloyd 1986). Then, a stigma-height mutation produces a reverse-herkogamous morph (one with the stigma lower than the anthers; fig. 1), which can become established in the population because of a frequency-dependent

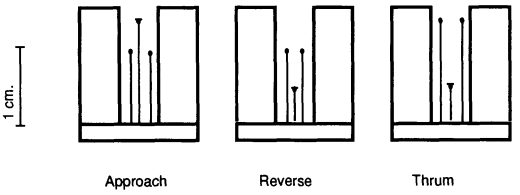

FIG. 1. Longitudinal sections through artificial flowers, demonstrating floral organ heights. Dimensions are given in text.

advantage in pollen transfer. Specific requirements for the establishment of a polymorphism depend on whether seed set is pollen-limited or resource-limited. For the resource-limited case, a polymorphism could become established if each morph donates pollen more successfully to flowers of the opposite morph than to flowers of its own morph. For the pollen-limited case, the sum of pollen donation and receipt between flowers of different morphs must be greater than twice the amount of intramorph transfer.

In a second mutation, anther position in the reverse-herkogamous morph is shifted to the height of the high stigmas. The population now consists of three morphs: approach, reverse, and thrum. The thrum could replace the reverse morph either because it is more efficient at cross-pollination with the approach morph, or because it has a lower selfing rate (if inbreeding depression is greater than 1/2). Similar inequalities are presumed to select for the lowering of approach anthers to the level of the thrum stigma and for ancillary features of distyly such as morph-specific stigmatic sculpturing. With the morphological polymorphism established, incompatibility groups evolve in response to selfing and inbreeding depression.

In families in which distyly has evolved, the morphology of nondistylous taxa supports the idea that approach herkogamy is a plausible ancestral condition. In a survey of 25 families in which heterostyly has arisen, the majority of species in all 25 have flowers with stigmas and/or anthers exserted from the floral tube (Lloyd and Webb 1992a). The majority of species in 22 of the 25 families have tubular corollas (Lloyd and Webb 1992a), a floral morphology known to co-occur with approach herkogamy (Webb and Lloyd 1986). Finally, in several cases cited by Lloyd and Webb (1992a), heterostylous taxa have close relatives with approach herkogamy.

Determining whether incompatibility groups or morphological distyly evolved first is a more difficult proposition, although the development of reliable phylogenies may eventually help. At present, we can at least determine the mechanical plausibility of the Lloyd-Webb model. A critical test of the model requires measuring pollen-transfer patterns among three floral types within one population: an approach-herkogamous (or simply "approach") ancestor, the initial reverse-herkogamous ("reverse") invader, and finally, the long-stamened "thrum" morph that supplants the reverse morph.

Natural populations provide limited opportunity for testing the model because none contains all three morphs. In a review of the Lloyd-Webb model, Barrett (1990) suggested that "experimental manipulation of the floral morphology of heterostylous and homostylous groups" would be necessary to resolve this problem. Satisfying though it would be, this approach presents obvious technical problems for most groups: delicate floral parts cannot simply be repositioned at will. Partial emasculations of flowers in a tristylous species could re-create the three flower types envisioned by the model; however, the source of pollen from midlevel anthers would be impossible to distinguish.

The use of artificial flowers to examine pollen-transfer patterns surmounts difficulties posed by attempts to work with natural populations. With artificial flowers, the position of the floral organs can be easily adjusted, and dyed pollen can be used to differentiate donor morphs. Having bumblebees visit the flowers allows us to include the most important biological determinants of pollen flow: the position the pollinators take relative to the reproductive organs, the specificity and reproducibility of pollen placement on their bodies, and the redistribution of pollen by grooming. If positions of anthers and stigmas can be found that produce the hypothesized patterns following pollinator visits, and if these positions are biologically reasonable, then the plausibility of the mechanism is demonstrated.

Artificial flowers can also be used to investigate the mechanism underlying disassortative pollination. Lloyd and Webb (1992b) suggested that the position of floral organs affects the free path taken by the proboscis as it probes the flower. We recorded videotapes of bees visiting artificial flowers with glass sides to investigate the effect of floral organ placement on pollinator feeding position.

In summary, the goals of the study were to measure pollen transfer by bees between artificial flowers to test the plausibility of Lloyd and Webb's (1992b) model. We compared pollen transfer first between approach-herkogamous and reverse-herkogamous flowers, and then between reverse-herkogamous and thrum flowers. We took video images of pollinators visiting all three flower types to demonstrate the effect of floral morphology on pollinator positioning within the flower and to investigate the mechanism underlying any nonrandom patterns of pollen transfer.

MATERIALS AND METHODS

Mechanics.—We kept a colony of bumblebees (*Bombus impatiens*) in a screened flight cage. The bees were allowed access to the outdoors through a small opening in the cage. Flowers were constructed of 1.5 cm-square, 1.4 cm-deep plexiglass blocks with 0.9-cm diameter holes drilled through the center, mounted on polyethylene bases (fig. 1). Floral organs were formed by inserting size 0 nylon-headed insect pins through the central well into the foam. Each flower contained two anthers and a style. We dipped anther pin heads into small dishes of dried *Narcissus* pollen, dyed with food coloring. The pollen adhered to the nylon pin heads by electrostatic attraction. Stigmas were made of 1.5 mm-square pieces of double-sided sticky tape, cut with a razor blade and attached to the central insect pin head. A series of jigs was used to set stigma and anther heights.

We arranged the flowers in a series of small arrays on a table in the flight cage (fig. 2) and pipetted 50% honey solution in the well of each flower. The central flower in each array had no pollen so that we could measure outcross pollen flow to it. Each set of arrays remained in the flight cage for 1 h, resulting in a single trial. Four trials were run each day for 7 d, with the positions of the arrays rotated in each trial. One morph bore red pollen and the other bore green pollen; these colors were switched back and forth on alternate days. To estimate the amount of pollen presented in these trials, we used an Elzone particle counter to count the amount of pollen placed on 15 each of red- and green-dyed anthers. We measured selfing rates by having individual bees visit one flower of each morph for a total of 30 trials. Red, green, and yellow pollen were rotated across the three flower types.

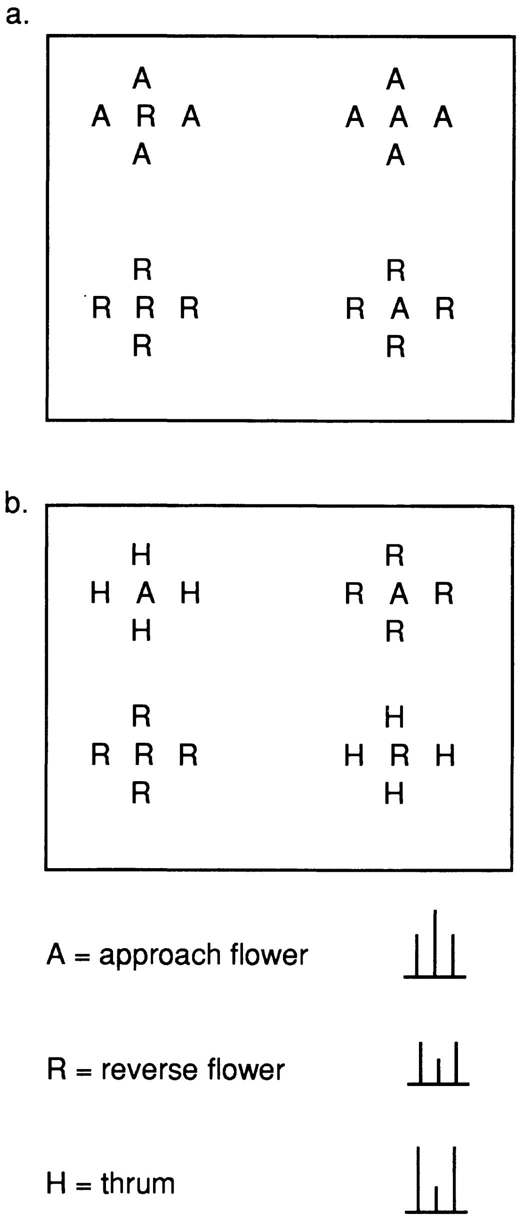

FIG. 2. a. Array arrangement for stigma-height polymorphism experiment. Placement of letters represents placement of flowers on table. The legend shows relative organ heights for each morph. The central flower in each subarray contains no pollen, so that outcross deposition can be measured. Flowers of different morphs bear different color pollen. Subarrays are rotated through the four positions at each trial. b. Array arrangement for anther-height polymorphism experiment. The top two subarrays allow comparison of thrum and reverse donation to approach flowers, whereas the bottom two allow comparison of thrum and reverse donation to reverse flowers.

Bee feeding behavior was recorded during a subset of trials. The time of landing, flower position, and time of departure from the flower were recorded for each flower visited from the time focal bees left the hive to the time they returned. For about half of these trials, 50 μl of nectar was added to each flower. For the other half, 25 μl was used.

After the arrays were removed from the flight cage, the pieces of tape were removed with clean forceps, mounted on slides, and covered with cover slips. The number of pollen grains of each color was counted with a dissecting microscope at 100×.

The first part of the experiment compared pollen transfer between approach-herkogamous flowers and reverse-herkogamous flowers (fig. 2a). Both types of flowers had stamens 9 mm high, whereas high stigmas were 13 mm and low stigmas were 5 mm. The second part compared reverse-herkogamous flowers and a "thrum" flower with stigmas at 5 mm and stamens at 13 mm (fig. 2b). The top two arrays in figure 2b. allow comparison of thrum and reverse donation to approach flowers, while the bottom two allow comparison of thrum and reverse donation to reverse flowers.

For the video work, we constructed artificial flowers with one glass side, and a 25 × 25 × 25-cm box with a clear plexiglass wall for conducting the trials. A table-mounted video camera at high magnification (10×) was focused on the flower, which filled the entire screen. We placed a previously trained, chilled bee on the flower, which contained 20 μl of 50% honey solution. We took footage of both small and large workers feeding on all three flower types, with floral organs arranged both perpendicular to, and in line with, the line of sight of the camera. The video image contained a timer including frame number (1 frame/2 s). We viewed the images frame by frame to record type of floral organ contacted (anther or stigma), duration of contact, and portion of bee's body making contact (distal, ventral, or lateral; proboscis, head, thorax, or abdomen).

Small bees contacted the flowers in a greater variety of ways; thus, only data from small bees are discussed here. We tested for lack of independence of floral morph, floral organ, and bee body part using a *G* test (Sokal and Rohlf 1981).

Testing Conditions of the Lloyd-Webb Model.—In each feeding bout, pollen from the donor morphs can be counted on the stigmas of each of the recipient morphs. These transfer probabilities are critical parameters in Lloyd and Webb's (1992a) model. Lloyd and Webb specified conditions for the invasion of new morphs in terms of a matrix of q_{ij}, defined as the number (quantity) of grains from morph *i* that are transferred on the stigma of morph *j* in successive visits to *i*, *j* flowers. The first stage of the model presents necessary conditions for a stigma-height polymorphism to become established, given presence or absence of pollen limitation of seed set. If seed set is solely resource-limited, these conditions must be met: $q_{\mathrm{ra}} > q_{\mathrm{aa}}$ and $q_{\mathrm{ar}} > q_{\mathrm{rr}}$, where a and r represent approach and reverse morphs, respectively. That is, donation by each morph to the opposite morph must be greater than donation to other plants of the same morph. Because pollen receipt is always sufficient for female function, only differential male function (i.e., donation success) is necessary for a polymorphism to establish. If seed set is pollen limited, $q_{\mathrm{ar}} + q_{\mathrm{ra}} > 2q_{\mathrm{aa}}$ and $q_{\mathrm{ar}} + q_{\mathrm{ra}} > 2q_{\mathrm{rr}}$ must both be fulfilled for the polymorphism to become established; i.e., morphs must on average transfer pollen more efficiently to and from flowers of the other morph than with flowers of their own type. Inspection of the data (table 2) indicated that there was substantial variation among different trials in the amounts of pollen deposited. To avoid pooling heterogenous data sets, we examined these inequalities within each trial and used sign tests to determine significant directionality of the inequalities across the entire set of trials (Sokal and Rohlf 1981).

Conditions for the establishment of the anther height polymorphism are somewhat more complicated. Pollen transfer is only part of the story, as morph-specific selfing rates and inbreeding depression are also important. Regarding the pollen-transfer components, it is assumed that the increase in anther height will cause the thrum morph to be less successful than the reverse morph at donating pollen to reverse flowers ($q_{\mathrm{rr}} > q_{\mathrm{hr}}$, where h represents the thrum morph). If this reduced success is more than offset by an increase in donation to the approach-herkogamous morph ($q_{\mathrm{ha}} > q_{\mathrm{ra}}$), then the anther-height polymorphism would be favored purely by pollen-transfer asymmetry. The difference between the two inequalities must be positive, indicating an increase in other-morph pollination that more than compensates for the reduction of within-morph pollination. Again, the experimental design allowed the comparisons of interest to be made for each trial. Sign tests were used to compare the number of trials in which the difference between the inequalities was positive to the number where the difference was negative, as well as to ascertain whether or not the inequalities themselves were validated.

TABLE 1A. Mean (sample size, standard error) number of seconds spent foraging per flower by bumble bees. A, approach-herkogamous flower; R, reverse-herkogamous flower; H, thrum-type flower. Flowers contained 25 or 50 μl nectar. Video flowers contained variable amounts of nectar, since re-filling between visits was not possible during filming.

	A	H	R
25 μl	31.8 (6, 16.32)	21.6 (22, 6.06)	21.6 (22, 3.84)
50 μl	150 (3, 66.90)	42.6 (15, 12.06)	43.2 (28, 9.90)
Variable	20.0 (27, 4.71)	12.4 (19, 2.36)	11.9 (22, 2.80)

TABLE 1B. Two-way ANOVA for mean foraging time/flower for flowers in arrays. Volume is amount of nectar in flower. Morph is approach, reverse, or thrum.

Source	df	SS	MS	F	pr > F
Volume	1	11.02	11.02	21.88	0.0001
Morph	2	7.41	3.70	7.35	0.001
Vol × morph	2	4.79	2.39	4.75	0.01
Error	90	45.30	0.50		

In the development of their model, Lloyd and Webb (1992b) assumed that self-deposition rates are equal among the approach- and reverse-herkogamous morphs ($u_a = u_r$). We examined this assumption by comparing single-visit selfing rates to all three morphs by individual bees. Wilcoxon signed-rank tests were used to compare selfing rates.

RESULTS

The majority of bee movements between flowers were between flowers on the same subarray (83.6%, $n = 72$, $P < 0.01$, G test).

Surprisingly, bees in arrays spent about twice as long foraging on flowers with high stigmas than on either of the morphs with low stigmas (table 1). Bees feeding on video flowers, where they were only presented with a single flower, also spent more time feeding on approach than on reverse or thrum flowers (table 1). Bee foraging behavior and the amount of pollen transfer also changed depending on the amount of nectar present. Larger nectar loads caused longer visits and less pollen deposition but not did not change the pattern of pollen transfer (table 1, fig. 3). Bees visited an average of 5.0 flowers on single foraging bouts if flowers contained 50 μl ($n = 9$, SE $= 0.83$) and 7.0 flowers if they contained 25 μl ($n = 7$, SE $= 0.76$; $P > 0.05$).

Pollen-bearing insect pins carried a mean of 4474.5 grains ($n = 22$, SD $= 1699.9$), which was independent of dye color. An average of 51.13 pollen grains per bout was found on the stigmas of the central, pollen-free flowers ($n = 216$, SD $= 45.67$). Because the ratio of pollen-bearing anthers to stigmas was 4:1, an average of 0.286% of the pollen originally on the anthers was delivered to stigmas. This fraction of production to deposition is within the range of estimates for natural systems (e.g., Ganders 1976; Thomson and Thomson 1989).

Across all trials, stigmatic pollen loads differed depending on the combination of donor and recipient (fig. 4). High stigmas received more pollen than low stigmas. Considering only inter-

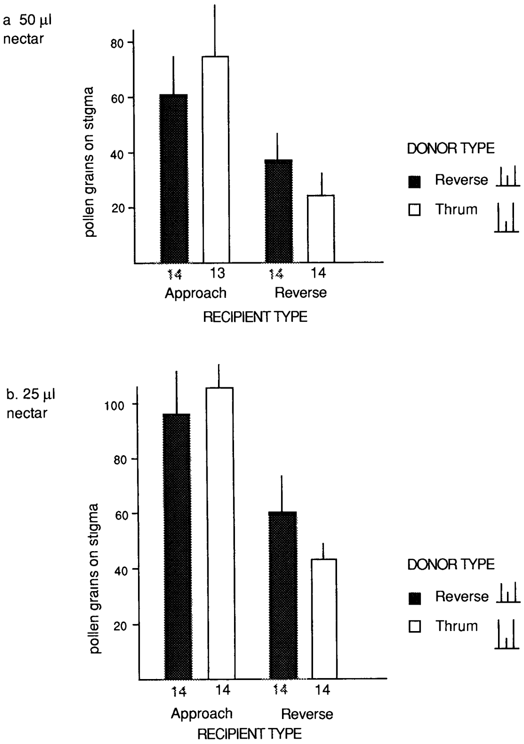

FIG. 3. Pollen grains transferred by bumblebees from reverse-herkogamous and thrum to approach- and reverse-herkogamous artificial flowers. Vertical lines indicate standard errors. Numbers below bars represent number of trials.

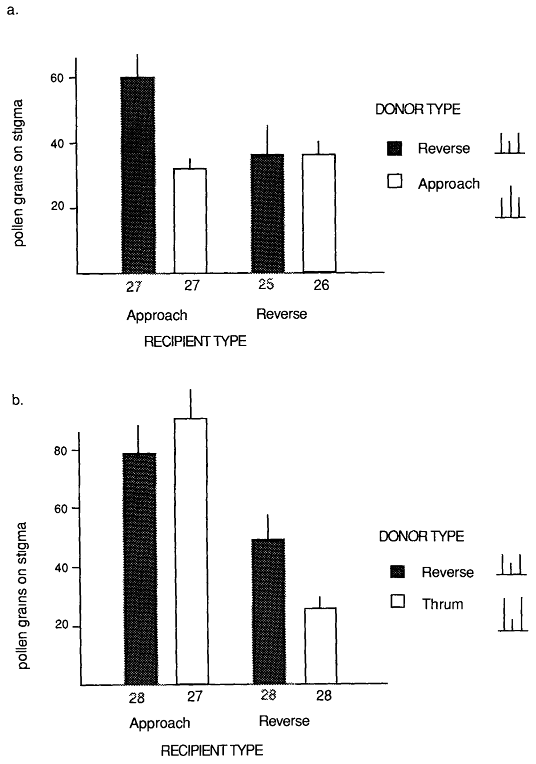

FIG. 4. a. Pollen grains transferred by bumblebees between approach-herkogamous and reverse-herkogamous artificial flowers. Vertical lines indicate standard errors. Numbers below bars represent number of trials. The values represented are means and obscure heterogeneity across trials. b. Pollen grains transferred from reverse and thrum to approach and reverse flowers.

TABLE 2. Pollen counts on stigmas. q_{aa} indicates number of grains from approach-herkogamous flowers that were counted on an approach-herkogamous stigma within a single trial, q_{ra} is the number of reverse grains counted on an approach stigma, and so on. Trials consisted of a set of four arrays visited by bees for 1 h (See fig. 2).

Trial	q_{aa}	q_{ra}	q_{ar}	q_{rr}
1	11	17	24	14
2	55	42	15	9
3	16	52	*	19
4	30	79	6	51
5	22	22	28	17
6	50	28	17	0
7	37	38	36	11
8	15	16	8	2
9	11	35	24	119
10	18	27	28	55
11	46	63	70	59
12	74	27	16	12
13	33	65	25	23
14	21	24	16	11
15	35	60	86	13
16	10	51	51	*
17	41	104	56	1
18	27	81	48	57
19	15	135	26	16
20	43	91	54	190
21	37	43	3	*
22	21	46	17	21
23	53	100	47	0
24	30	65	53	32
25	14	72	82	69
26	74	165	93	60
27	23	89	45	21
Mean	31.9	60.6	36.1	32.7
SD	17.58	35.55	24.92	40.71

* Missing values.

morph transfer, high stigmas received an average of 60.6 to 88.2 grains, depending on the donor type (fig. 4, tables 2, 3). In contrast, intermorph donation to low stigmas averaged only 25.1 to 36.1 grains (fig. 4, tables 2, 3).

The videos revealed that bees' bodies contacted floral organs in a structured way (table 4). Tall organs (stigma on approach flowers, anthers on thrums) tended to contact the thorax and abdomen, whereas short stigmas and anthers contacted primarily the head and thorax (fig. 5). Style position affected dorso-lateral positioning of the bee such that some floral organs for some morphs tended to be contacted laterally, whereas others tended to be contacted dorso-ventrally (fig. 6).

The plausibility of the evolution of a stigma-height polymorphism was upheld by the pollen-transfer levels that we found. Of the inequalities required for the resource-limited case, $q_{ra} > q_{aa}$ was satisfied in 23/27 trials ($P < 0.01$, sign test), and $q_{ar} > q_{rr}$ was satisfied in 18/24 trials ($p < 0.05$; sign test). In other words, both morphs were more successful at donating pollen to flowers of the opposite morph than to flowers of their own morph. Considering the pollen-limited case, $q_{ar} + q_{ra} > 2q_{aa}$ was satisfied in 20 of 27 trials ($P < 0.05$; sign test), and $q_{ar} + q_{ra} > 2q_{rr}$ was satisfied in 20 of 24 trials ($P < 0.01$, sign test). Thus, the sum total of intermorph transfer was greater than twice the intramorph transfer for either morph, establishing conditions for a protected polymorphism if both female and male reproductive success are limited by pollen transfer.

In contrast, the establishment of an anther-height polymorphism, based on pollen-transfer patterns alone, was only partially supported by the data. One of the necessary inequalities was

TABLE 3. Pollen counts on stigmas. q_{ra} indicates number of grains from reverse-herkogamous flowers that were counted on an approach-herkogamous stigma within a single trial, q_{ha} is the number of thrum grains counted on an approach stigma, and so on. Trials consisted of a set of four arrays visited by bees for 1 h (fig. 2). Flowers in trials 1–16 contained 50 μl of nectar. Flowers in trials 17–28 contained 25 μl of nectar.

Trial	q_{ra}	q_{ha}	q_{rr}	q_{hr}
1	149	41	78	31
2	134	59	26	112
3	97	144	68	67
4	137	*	27	55
5	34	105	3	12
6	2	73	19	29
7	25	27	6	3
8	0	11	16	10
9	25	99	87	13
10	0	0	1	6
11	104	104	13	0
12	57	36	7	0
13	7	44	53	10
14	71	232	100	22
15	61	122	30	10
16	63	108	68	61
17	33	114	106	18
18	101	150	43	22
19	135	110	84	2
20	123	138	201	80
21	30	43	7	26
22	37	93	0	32
23	69	96	9	29
24	71	61	54	2
25	142	137	47	22
26	35	138	37	20
27	206	103	94	0
28	219	82	65	10
Mean	77.4	88.2	48.2	25.1
SD	60.36	52.58	44.78	27.06

* Missing values.

upheld: $q_{rr} > q_{hr}$ for 20/28 trials ($P < 0.05$, sign test). The other was not: $q_{ha} > q_{ra}$ for only 17/27 trials ($P > 0.05$, sign test). Although the median value for q_{ha} was 99 and the median for q_{ra} was 61, the distributions of the values were not significantly different (Mann-Whitney *U* test). Therefore, the increase in donation to the approach morph exceeded the loss in donation to the reverse morph but not significantly. These results do not refute the possibility of spread of an anther-height mutation, but neither do they provide strong support.

However, pollen-transfer probabilities are not the only parameters needed to predict the likelihood of establishment of a thrum-type variant. Morph-specific selfing rates and inbreeding depression are also important (Lloyd and Webb

TABLE 4. Log-linear model for number of contacts by bee body parts to floral organs for three types of artificial flower. Morph is approach, reverse, or thrum. Floral organ is anthers or stigma. Bee body part is head, thorax, or abdomen. Number of contacts were summed over 27 visits to approach flowers, 22 visits to reverse flowers, and 19 visits to thrum flowers.

Effect	*G*	df	*P*
Morph × floral organ × body part	181.24	12	.001
Morph × floral organ	66.74	3	.001
Morph × body part	37.18	4	.001
Floral organ × body part	17.66	3	.001

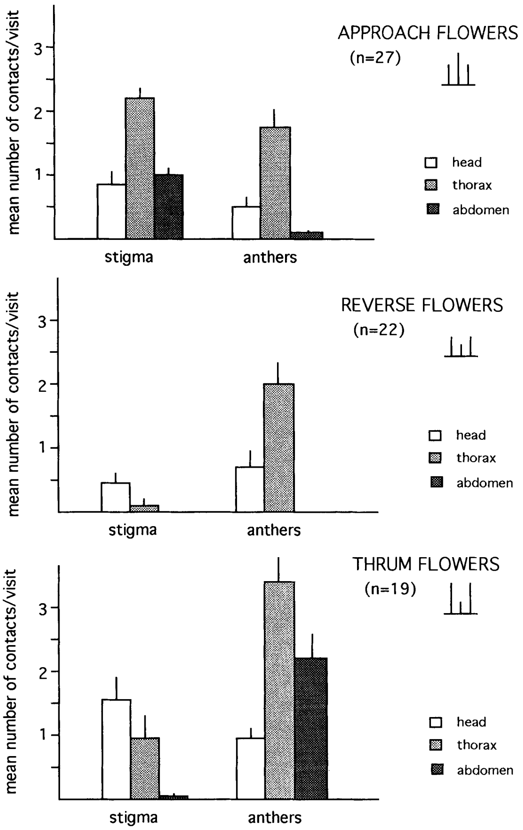

FIG. 5. Mean number of contacts with anthers and stigmas by various body parts of bees visiting three types of artificial flowers. Approach flowers have high stigmas and midheight anthers, reverse flowers have low stigmas and midheight anthers, and thrum flowers have low stigmas and high anthers. Vertical lines indicate standard errors. Sample size refers to number of visits.

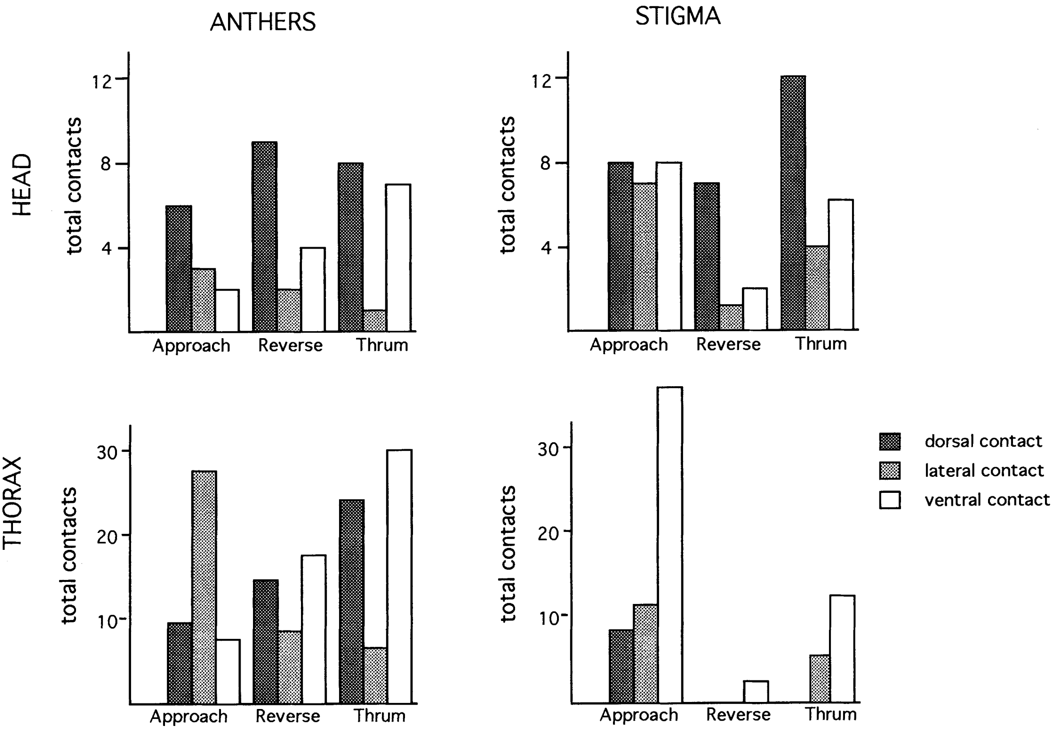

FIG. 6. Orientation of bees' heads and abdomens as they contacted anthers and stigmas of three types of artificial flowers. Approach flowers have high stigmas and midheight anthers, reverse flowers have low stigmas and midheight anthers, and thrum flowers have low stigmas and high anthers. Values are total number of contacts from 27 visits to approach flowers, 22 visits to reverse flowers, and 19 visits to thrum flowers.

1992b). For example, if inbreeding depression is weak, a reduction in self-pollination by the new morph would decrease its likelihood of spreading in the population. If inbreeding depression is strong, the thrum morph could replace the reverse morph if it had a lower selfing rate. Selfing rates did not differ for the three morphs. Mean per-visit selfing rates were 28.1 for the approach morph (n = 31, SE = 4.72), 31.0 for the reverse morph (n = 31; SE = 3.87), and 32.6 for the thrum morph (n = 26; SD = 6.39). We did not find differential selfing rates that might increase support for the second stage of the model.

DISCUSSION

Evolutionary Interpretation.—Bumble bees transferred pollen among these flowers in patterns that reinforce the plausibility of a reverse-herkogamous variant being able to invade an ancestral population of approach-herkogamous flowers. This is perhaps the most important aspect of the Lloyd-Webb model, and the aspect that differs most from the viewpoint of Charlesworth and Charlesworth (1979). In the scenario envisioned by the Charlesworths, with a non-herkogamous ancestor a style-length polymorphism is unlikely to be favored by selection for pollen receipt. Lloyd and Webb came to the opposite conclusion by considering pollen donation (male function) and invoking a herkogamous ancestor. Of this crucial step in the Lloyd-Webb scenario, Barrett (1990) commented that "While it is well known that even low levels of disassortative mating result in polymorphic equilibria in self-compatible populations, it is less clear whether a simple change in stigma height is likely to give rise to this pattern of mating." Our results certainly demonstrate that such disassortative mating is possible. That we obtained those results with the first flower design chosen might even indicate that it is likely, although further exploration of various artificial morphologies would be necessary for a formal assessment of likelihood. Our results also suggest that selection through male function is sufficient, whether or not selection through female function is operating, that is, whether seed production is pollen-limited or resource-limited.

The second stage of the Lloyd-Webb model, the evolution of an anther-height dimorphism, was seen by Lloyd and Webb as "easy to achieve" after the stigma-height dimorphism has evolved. Barrett (1990) also raised no objection to this stage of the model, in contrast to his agnostic position (above) regarding the establishment of the stylar dimorphism. For our design of flowers, however, support for this phase is weak. Although the pollen-transfer patterns went in the right direction more often than not, this tendency fell short of significance.

In contrast to the first stage, in which interference between style and stamens dictates that only a mutation of large effect would make a difference, the second stage in the evolution of full distyly might be more likely to proceed from a series of small changes in anther height occurring because of performance advantages for flowers with anthers more similar in height to opposite-morph stigmas. If this is the case, it is to be expected that selection pressures on the second stage are less strong and would not be revealed without larger sample sizes. The greater separation between stigma heights than anther heights in many species supports this possibility (e.g., *Anchusa officinalis,* Phillip and Schou 1981; *Psychotria spp.,* Hamilton 1990).

Robustness of Pollen-Transfer Inequalities.—We chose the single feeding trial as our unit of comparison, because there was considerable heterogeneity of pollen deposition across trials. If pollen-transfer probabilities are pooled across trials, some of them are more robust than others. In general, all of the patterns hold, with the exception of inequalities involving pollen transfer between reverse flowers (q_{rr}). Figure 4 shows that the mean for $q_{ar} = q_{rr}$, in contrast to the sign-test results, which show $q_{ar} > q_{rr}$. Examination of table 2 shows that several extremely high values for q_{rr} are responsible for the difference between the two types of comparisons. Using the Wilcoxon signed-ranks test, $q_{ar} > q_{rr}$ at $P < 0.06$, very close to the conventional criterion for statistical significance.

Which statistical approach is most appropriate? Stigmatic pollen loads in our design are analogous to lifetime floral stigmatic pollen loads in nature. Means are not appropriate, because they imply that excess pollen donated to one flower can be transferred to another. If seed set is resource-limited (as in this comparison), the number of flowers for which a morph wins the majority of ovules should be more important than the magnitude of differences between flowers in which it was in the majority versus the minority. The sign test is more appropriate than the signed-ranks test under these conditions. The results hold, although it is worth noting that inequalities involving approach flowers as recipients are more robust than those involving them as donors. This pattern is consistent with the video footage and with observations from natural flowers discussed below.

TABLE 5. Mean and median number of pollen grains transferred between two morphs. q_{ra} indicates number of grains from reverse-herkogamous flowers counted on an approach-herkogamous stigma, q_{ha} is the number of thrum grains counted on an approach stigma, and so on. Separation is the distance (mm) between the anther height of the donor and the stigma height of the recipient.

	q_{ha}	q_{ra}	q_{rr}	q_{ar}	q_{aa}	q_{hr}
Mean	88.2	69.2	39.6	36.1	31.9	25.1
Median	99	61	26	28	30	19
Separation	0	4	4	4	4	8

Mechanism.—In general, the results confirm Darwin's (1877) prediction that pollen transfer would be most efficient between anthers and stigma presented at the same height. The median transfer values were lowest between flowers in which anthers and stigma were 8 mm apart, highest between flowers with anthers and stigma at the same height, and intermediate between flowers in which anther and stigmas were 4 mm apart (table 5). Video images of bees contacting floral parts demonstrate that contacts between long and short floral organs are segregated on bees' bodies, in accordance with studies of wild-caught pollinators of heterostylous plants (Darwin 1877; Olesen 1979; Lewis 1982).

For the stylar polymorphism to become established, however, the position of the stigma of the invading morph must create asymmetrical pollen flow in the absence of an anther-height polymorphism, that is, when anther-stigma height separation does not differ between intra- and intermorph pollinations. What is the mechanism for this asymmetry that we observed? Lloyd and Webb (1992b) postulated that the "free path" taken by the proboscis of the pollinator differs between the two morphs. They suggested that the positions of the floral organs constrain the path available for probing by the pollinator, affecting which portions of the proboscis come into contact with floral organs. The presence or ab-

TABLE 6. Probability of individual pollen grains being transferred between and within morphs in distylous species. Probability calculated from $T_{ij} = r_{ij}f_j/p_if_i$, where T is the probability of a pollen grain produced by morph i landing on a stigma of morph j, r is the mean stigmatic pollen load for this transfer type, p_i is the pollen production of the donating morph, and f_i, f_j are the population frequencies of the donor and the recipient (equation from Lloyd and Webb 1992b). All values are multiplied by 10^{-3}. The superscript ^{E} indicates that recipients were emasculated; intramorph values without this superscript are included for comparative purposes but are not actual transition probabilities, since they include self pollen. Sample sizes following the reference indicate that the values represent means from a number of populations or years.

Species	T_{pp}	T_{tp}	T_{pt}	T_{tt}	Reference
Amsinckia douglasiana		3.9	2.4	1.3	Ganders 1976
			2.4^E	0.7^E	
A. grandiflora		3.7	3.1		Ornduff 1976 (n = 3)
A. vernicosa var. *furcata*		4.0	10.8		Ganders 1976
Cratoxylum formosum		6.1	0.4		Lewis 1982*
Hedyotis caerulea		36.1	31.0		Ornduff 1980a (n = 12)†
Hypericum aegypticum		8.2	4.0		Ornduff 1975
Jepsonia heterandra	3.0^E	4.5^E	2.7^E	1.3^E	Ganders 1974
	13.4	7.7	2.7	2.1	
J. parryi		0.7	0.7		Ornduff 1970‡
Lithospermum caroliniense		1.4	0.5		Weller 1980, 1985 (n = 9)§
Lythrum californicum		12.8	4.2		Ornduff 1978 (n = 6)
L. lineare		58.1	1.6		Ornduff 1978‖
Primula elatior		10.3	5.0	4.0	Schou 1983#
			5.5^E	4.0^E	
P. veris		2.1	1.7		Ornduff 1980b (n = 3)
P. vulgaris		1.6	0.7		Ornduff 1979 (n = 4)
P. vulgaris	1.0^E	3.1^E	0.8^E	1.7^E	Piper and Charlesworth 1986**
	13.0	3.0	0.7	6.8	
Pulmonaria obscura		1.7	1.9		Olesen 1979††
Turnera subulata		6.2	2.1		Rama Swamy and Bahadur 1984 (n = 5)‡‡

* Assumed pin : thrum ratio 50:50. Stigmatic pollen load values taken from abstract.

† Morph ratio data taken from 12 populations for which stigmatic pollen loads were available. Pollen production data taken from 4 populations reported.

‡ Morph ratios are means from 8 populations.

§ Mean stigmatic loads/population were first calculated (combining data from old versus young flowers and/or small versus large plants). These were pooled, excluding a Miller Dunes 1978 report (Weller 1980) since data from this population were included in both references. Pollen production and morph ratios are means from three populations (Weller 1980).

‖ Pollen transfer values strongly influenced by a pin : thrum morph ratio of 0.85:0.15 based on a sample size of 20 plants.

\# At 12 d after anthesis, when stigmatic pollen loads were at a maximum.

** The ratio of plants in the population was 50:25:25 pin : thrum : long-homostyle. Long-homostyles possessed thrum-type pollen. Therefore, I have assumed that the p:t ratio for thrums as donors is 50:50, but the ratio for pins as donors is 50:25.

†† A sample of the stigmatic surface was counted.

‡‡ Assumed pin : thrum ratio 50:50.

sence of the long style leads to different patterns of pollen pickup, even though anther heights do not differ between the two morphs.

Videotapes provide evidence that the long style does dictate bee posture in such a way that pollen from long-styled morphs is picked up on body regions distinct from the areas where pollen from short-styled morphs is picked up. Bees feeding on approach flowers tend to enter the flower so that the long style lies along the midline of the body, with anthers contacting the thorax laterally (fig. 6). Bees feeding on reverse or thrum flowers, in contrast, often enter perpendicularly, so that the anthers contact primarily the ventral or dorsal thoracic regions (fig. 6). Stigma contacts are also affected by bees' posture. Bees contact approach stigmas predominantly with the ventral surface of the thorax (fig. 6), providing an excellent mechanism for pollen transfer from reverse and thrum to approach flowers. The mechanism for transfer from approach to reverse flowers is not so apparent. Bees contacted reverse stigmas relatively infrequently, and predominantly with the dorsal side of the head, an area which contacted anthers of all flower types in similar proportions (fig. 6).

Comparisons with Natural Flowers.—Pollen-transfer patterns for the artificial flowers are consistent with those reported from natural populations. Studies of open-pollinated stigmas allow a comparison of pollen flow between morphs because of the pollen-size polymorphism associated with distyly in many species (Ganders 1979; Dulberger 1992). In 13 of 17 cases surveyed, pollen flow was greater from thrum to pin flowers than from pin to thrum flowers (table 6). This result reinforces a general impression that pollen transfer is higher between high anthers and long stigmas than between organs placed deep within the corolla tube (Levin and Berube 1972; Beach and Bawa 1980).

Tristylous species have similar pollen-transfer patterns. In *Pontederia cordata,* midlevel anthers to mid stigmas have the highest transition probability of any of the nine possible combinations in five of nine experiments, and long anthers to long stigmas have the highest probability in three of nine experiments (Harder and Barrett unpubl. calculations; data from Price and Barrett 1982, 1984; Barrett and Glover 1985; Glover and Barrett 1986). The midheight organs in *Pontederia* are placed near the mouth of the corolla, implying that this may be the optimal position for both pollen donation and receipt. However, as Harder and Barrett pointed out, the transfer probabilities are strongly influenced by the ratio of the morphs in the population. Measurement of pollen stigmatic loads following known sequences of visits, or construction of populations with equal morph ratios would be the best way to measure transfer probabilities.

With experimental populations of the self-compatible tristylous *Eichhornia paniculata,* Kohn and Barrett (1992a) found that the short-styled morph had the highest male fitness and the long-styled morph was relatively female. Subsequent experiments (Kohn and Barrett 1992b) revealed that high-level anthers donated pollen to compatible stigmas more efficiently than low-level anthers. In *Lythrum salicaria,* O'Neill (1992) found that dye transfer among morphs was asymmetric, with more than random expectation of transfer from short-styled morphs to mid- and long-styled morphs. Once again, pollen transfer between higher-level organs predominates. These experiments support the argument for the introduction of a reverse-herkogamous mutant in order to increase male fitness.

Toward Increasing Realism.—Our artificial flowers demonstrated that the presence or absence of a long style can affect the pollinator's stance in a stereotypical way, resulting in disassortative pollination between morphs. How robust is this result, and how dependent is it on the particular floral design we chose? The size and anther-stigma separation in our flowers is within the range found in nature (Ganders 1979); however, several other features could be modified to more closely approximate natural flowers. First, the sides of the corolla were parallel, instead of tapering towards the base. It is probable that a tapering corolla would increase contact with the low stigma on reverse and thrum flowers. Second, the insect pins used for floral organs are much stiffer than natural styles and filaments, perhaps dictating pollinator posture more extremely. Third, the unrealistically high nectar volumes used to induce bees to feed resulted in unnaturally long visits. Prototype flowers that avoid these drawbacks are in the works. It would be interesting to construct a series of such flowers to see how well asymmetrical pollen flow persists over a range of morphologies.

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