EXPERIMENTAL STUDIES ON THE FUNCTIONAL
SIGNIFICANCE OF HETEROSTYLY

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Abstract. —Heterostyly has been viewed as both an antiselfing device and a mechanism that increases the proficiency of pollen transfer between plants. We used experimental manipulation of the morph structure of garden populations of self-compatible, tristylous *Eichhornia paniculata* to investigate the function of floral polymorphism. Outcrossing rates (t), levels of intermorph mating (d), and morph-specific male and female reproductive success were compared in replicate trimorphic and monomorphic populations. In trimorphic populations, t and d averaged 0.81 (2 SE = 0.03) and 0.77 (2 SE = 0.03) respectively, with no difference in either parameter among morphs. Ninety-five percent of outcrossed seeds were therefore the result of intermorph fertilizations. Male reproductive success of the long-styled morph was low, especially in comparison with plants of the short-styled morph. Outcrossing rates for each morph were higher in trimorphic than monomorphic populations where t averaged 0.71 (2 SE = 0.01), 0.30 (2 SE = 0.04) and 0.43 (2 SE = 0.1) for the long-, mid-, and short-styled morphs, respectively. Seed set was lower in monomorphic populations, particularly those composed of the L morph, reflecting reduced pollen deposition. Floral polymorphism therefore increased both outcrossing rate and fecundity but the magnitude of the differences varied among morphs. If the ancestral condition in heterostylous groups resembled the L morph, as has been suggested, data from this study suggests that the selective basis for the establishment of floral polymorphism could have been increased pollen transfer rather than higher levels of outcrossing.

Key words. —*Eichhornia paniculata*, experimental manipulations, functional significance, gender measures, heterostyly, marker genes, pollen transfer, reciprocal herkogamy.

Heterostyly provides a remarkable example of the convergent evolution of morphological and physiological traits found in at least 25 diverse flowering plant families. Two major features characterize this genetic floral polymorphism: 1) the reciprocal positioning of stigmas and anthers among the floral morphs (hereafter called reciprocal herkogamy), and 2) a self- and intramorph incompatibility system (heteromorphic incompatibility) in which only pollen from the same level as the stigma is compatible. These features have led to the view that heterostyly functions as both an antiselfing device and a mechanism that increases the proficiency of pollen transfer between plants. Models for the evolution of heterostyly can be distinguished by the emphasis they place on each of these functions (reviewed in Barrett, 1990).

Charlesworth and Charlesworth (1979), and Ganders (1979), proposed that inbreeding depression promoted the evolution of heteromorphic incompatibility as a selfing avoidance mechanism. Reciprocal herkogamy then arose secondarily to promote pollen transfer between the incompatibility types. However, while heteromorphic incompatibility prevents self-fertilization, it also prevents intramorph mating. Because of the small number of mating groups (two in distyly, three in tristyly), the cost of incompatibility, in terms of reduced numbers of potential mates, is severe relative to plants with homomorphic multiallelic incompatibility systems.

This observation led Darwin (1877), and more recently Lloyd and Webb (1992a, 1992b), to suggest that heterostyly is unlikely to have evolved primarily as a selfing avoidance mechanism. Instead they suggest that reciprocal herkogamy evolved prior to incompatibility as a result of selection on the male component of fitness (and see Charlesworth, 1979). The initial step was the evolution of an alternative floral morphology that resulted in increased pollen transfer between unlike individuals. In Darwin's view, incompatibility developed secondarily as a pleiotropic by–product of se-

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lection for increased pollen competitive ability on the style type to which pollen was most frequently transferred. In contrast, Lloyd and Webb (1992b) argue that heteromorphic incompatibility reduces selfing, but arises when most interplant pollen transfer is already intermorph as a result of reciprocal herkogamy. Thus the cost of self-sterility, in terms of lost ability to fertilize plants of the same incompatibility type, is reduced.

Most heterostylous species exhibit both reciprocal herkogamy and heteromorphic incompatibility making it difficult to measure the independent effects of each feature of the polymorphism on reproduction. The direct effects of reciprocal herkogamy on mating patterns of the floral morphs can be assessed using self-compatible heterostylous plants (Ganders, 1975; Barrett et al., 1987). Self-compatibility allows comparison of reproductive parameters in populations in which floral morph structure is manipulated experimentally. In so doing, one can isolate the reproductive consequences of reciprocal herkogamy from those of heteromorphic incompatibility as well as from the effects of each morph's particular floral architecture in the absence of floral heteromorphism.

Reciprocal herkogamy may also affect mating patterns by imposing constraints on the performance of morphs as male and female parents. The stigma of the long-styled morph is exserted beyond the level of the anthers while, in the short-styled morph, the reverse is true. If long-level organs are better able to contact pollinators, the efficiency of transfer may exceed the efficiency of transfer between short-level organs. The long-styled morph may therefore perform better as a female parent while the short-styled morph may attain more reproductive success through male function. This has been hypothesized to lead to gender specialization, morph-specific differences in allocation to male vs. female structures, and the evolution of separate sexes (Willson, 1979; Beach and Bawa, 1980; Casper and Charnov, 1982; Charnov, 1982; Taylor, 1984; Charlesworth, 1989). Quantitative estimates of gender in self-compatible heterostylous populations can be obtained by the use of genetic markers allowing the measurement of morph-specific male and female reproductive success.

In this study we use genetic markers and experimental trimorphic and monomorphic populations of self-compatible, tristylous *Eichhornia paniculata* to address the following questions concerning the effects of reciprocal herkogamy: 1) Are outcrossing rates higher in trimorphic populations than in those composed of a single morph? 2) In trimorphic populations, how effective is reciprocal herkogamy at promoting intermorph (disassortative) mating? 3) How does the morph structure of populations influence seed set and are differences the result of variation in pollen transfer? 4) In monomorphic populations, what is the influence of morph-specific floral architecture on levels of outcrossing and seed set? 5) In trimorphic populations, are there morph-specific difference in gender?

This study uses experimental garden populations of the neotropical, annual, emergent aquatic *Eichhornia paniculata*. In the center of its range in northeast Brazil, *E. paniculata* is pollinated primarily by long-tongued solitary bees (*Ancyloscelis* and *Florilegus* spp.; Husband and Barrett, 1992). For logistical reasons the experiments described here were performed in southern Ontario where bumblebees (predominantly *Bombus fervidus, B. impatiens,* and *B. vagans*) were used as surrogate pollinators. These experiments evaluate the effects of heterostyly under these artificial conditions, rather than test how the polymorphism affects mating in natural populations. An important issue therefore, is to what extent the functioning of heterostyly depends upon the precise plant-pollinator relationship. We address this question by comparing mating patterns in experimental populations with those measured in northeast Brazil.

MATERIALS AND METHODS

Plants of *Eichhornia paniculata* used in these experiments were produced from controlled crosses among individuals grown from approximately 50 open-pollinated families collected from population B46 growing in a low-lying pasture near Quixadá, Ceará state, northeast Brazil. Details of the reproductive biology of *E. paniculata* can be found in Barrett (1985) and Morgan and Barrett (1989) and data on population genetic parameters for population B46 are given in Barrett and Husband (1990). We

made 361 crosses among 214 individuals to keep plants used in the experiments as unrelated as possible. No more than four plants used in any experimental population were half or full sibs. To enable joint estimates of outcrossing rates and levels of intermorph mating, plants were crossed to produce individuals homozygous at the triallelic *AAT-3* locus and at one or, usually, both of two diallelic loci, *PGI-2* and *AcPh-1*. All plants were assayed electrophoretically twice to confirm genotypes at these three loci.

Experimental populations contained 36 plants, either all the same morph (monomorphic), or 12 of each morph (trimorphic) placed approximately 30 cm apart in a six by six grid in a garden at Etobicoke, Ontario. Plants were assigned positions within arrays at random and exposed to pollinators for a single day. The four experimental treatments [trimorphic, monomorphic long-styled (L) morph, monomorphic mid-styled (M) morph, monomorphic short-styled (S) morph] were replicated three times each between July 27 and Aug 19, 1989 in a randomized complete block design. Approximately one third of all plants used were present in more than one array of the same treatment.

For all plants in experimental populations we counted the number of open flowers (flowers last 6 hr and each plant had a single inflorescence) and later recorded the number of fruits set. Seeds were then counted from five fruits per plant (fewer if less than five were available) and five seeds (occasionally fewer) from each of two fruits per plant were assayed electrophoretically for mating system analysis (see below).

To determine levels of pollinator activity among experimental treatments, we observed each population for three 10-minute periods, one each hour for the first three hours after pollinators were first observed foraging on experimental populations. During each observation period we recorded the species of the predominant pollinator (*Bombus* spp.), the amount of time they foraged in the population, and the number of flowers visited. Other insect visitors and the number of flowers visited were also noted. To determine the number of pollen grains deposited on stigmas, one stigma from each plant in one replicate per treatment was pre-served in 70% alcohol and the number of grains later counted under a compound microscope after staining with 5% basic fuchsin.

In trimorphic populations, plants of each morph were homozygous for one of three *AAT-3* alleles and the morph carrying each allele was varied among replicates. Thus a seed heterozygous at *AAT-3* indicated an intermorph mating and the identity of the paternal floral morph could be determined. Within floral morphs, plants were chosen so that alternative homozygous genotypes at *PGI-2* and *AcPh-1* were as evenly represented as possible. Outcrossing rates based on all three isozyme loci were estimated using the multilocus outcrossing rate (MLT) program of Ritland (1990, see also Ritland and Jain, 1981). The rate of intramorph outcrossing was calculated as the difference between the multilocus outcrossing rate and the proportion of seeds sired through intermorph matings. In monomorphic populations, approximately one third of the plants were homozygous for each *AAT-3* allele and outcrossing rates were estimated as above using all three loci. Means and standard errors of mating parameters were calculated from the morph-specific values of each parameter for the three replicates of each treatment.

Electrophoretic procedures used were modified from Glover and Barrett (1987) in the following way: Seeds were assayed directly by crushing in two drops of buffer (20 mg diethyl-dithio-carbamic acid, 10 mg EDTA (mol. wt. 292.2), 25 mg BSA, and one drop Tween 80 dissolved in 20 ml of 0.05 M sodium phosphate pH 7.0). The slurry was absorbed onto very thick filter paper wicks (Whatman's #17) and inserted into 11% starch gels.

Using the identity of the morph that sired each seed in trimorphic populations and the number of seeds produced by each plant, we calculated the functional gender (successful female gametes/total successful gametes), and total outcrossing rate (male and female gametes contributed to outcrossed offspring/total successful gametes) for each morph in each trimorphic population (see appendix for equations). We contrasted these values with estimates that assume male fitness is proportional to the relative number of polliniferous flowers produced (i.e., mea-
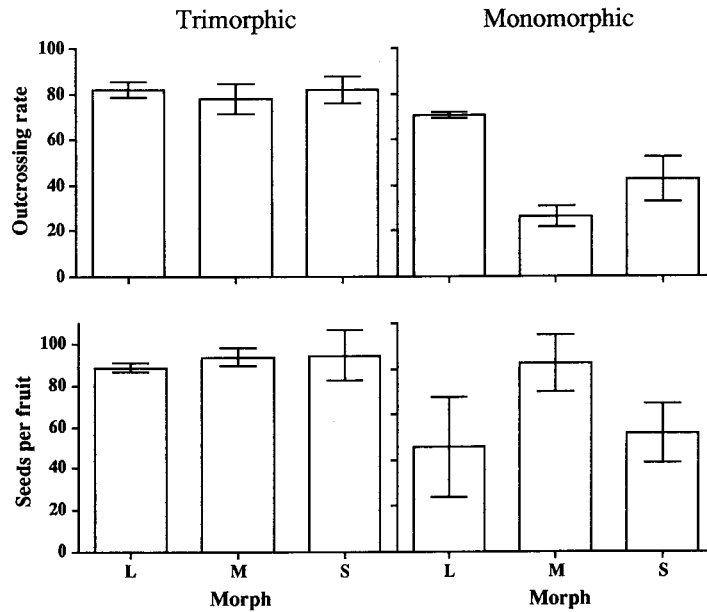
FIG. 1. Mean morph-specific outcrossing rates and seed set in experimental trimorphic and monomorphic populations of *Eichhornia paniculata*. Bars represent the range of mean values from the three replicates of each treatment.

sures such as those of Lloyd, 1980). Such measures have been used to estimate gender in studies where male fitness could not be measured directly (e.g., Thomson and Barrett, 1981; Devlin and Stephenson, 1987; Delesalle, 1989).

To assess the seed fertility of self-, intramorph, and intermorph pollen, hand pollinations were performed in a pollinator-free glasshouse on 12 plants of each morph chosen from those used in the experimental populations. Pollinations were performed by evenly coating stigmas with pollen from a single anther. For self- and intramorph pollinations of the L and S morphs, the anther level closest to the stigma was used, whereas for the M morph, pollen from long-level anthers was used. Pollen for intermorph pollinations came from the same level as the stigma (legitimate pollen). Two flowers on each plant were pollinated with each pollen type. Pollinations were performed on two plants per morph on each of six days. Each day, two pollen donors from each morph were used. Pollen for the two intramorph pollinations on each plant came from different donors and pollen for each of the intermorph pollinations came from different morphs. Seed set was analyzed us-

ing mixed model ANOVA with morph and cross type as fixed effects and plant as a random effect (SAS Institute Inc., 1988).

RESULTS

Pollinator visitation. —*Bombus* spp. were by far the most common visitors to experimental populations with workers of *B. fervidus* and *B. vagans* the most abundant. Occasional visits by butterflies, bee-flies, and sweat bees (*Agopostemon* spp.) accounted for less than 1% of the total visits observed. Visitation rates by *Bombus* spp. varied more than sevenfold across populations (total seconds $\bar{x}$ = 913, range 254–1,511; number of visits $\bar{x}$ = 270, range 61–442), apparently due to daily fluctuations in temperature and wind speed. There was no significant effect of experimental treatment on either the amount of time bumblebees foraged ($F_{3,8}$ = 0.39, P > 0.75), or the number of flowers visited ($F_{3,8}$ = 0.56, P > 0.5).

Mating System Analyses. — In trimorphic populations, outcrossing rates (t) averaged 0.81 (2 SE = 0.03) and rates of intermorph mating (d) averaged 0.77 (2 SE = 0.03) with no difference in either parameter among morphs (t, $F_{2,4}$ = 0.45, NS; d, $F_{2,4}$ = 0.98, NS) or replicates (t, $F_{2,4}$ = 0.06, NS; d, $F_{2,4}$
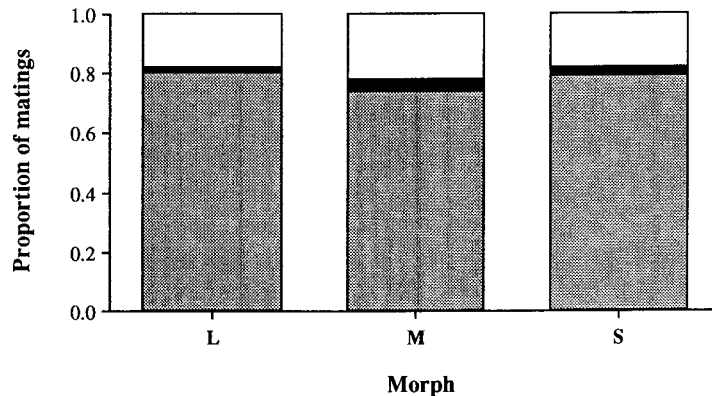
FIG. 2. Proportion of seeds produced from self-, intramorph, and intermorph mating for each morph in experimental trimorphic populations of *Eichhornia paniculata*. Open bars represent self, solid bars represent intramorph, stippled bars represent intermorph.

= 0.35, NS; Figs. 1, 2). Hence 95% (0.77/ 0.81) of outcrossed seeds resulted from intermorph fertilizations. If outcrossing events were random, expected rates of intermorph mating would be 69% because 24 of 35 non-self pollen donors in each array are a different morph than the recipient. Thus the outcrossed fraction of matings was highly disassortative.

In monomorphic populations, outcrossing rates averaged 0.71 (2 SE = 0.01), 0.30 (2 SE = 0.04) and 0.43 (2 SE = 0.1) for the L, M, and S morphs, respectively. Differences between floral morphs were significant ($F_{2,6}$ = 56.8, P < 0.001), and each morph had a lower outcrossing rate in monomorphic than trimorphic populations (t-tests, DF = 4; L, t = 8.21, P < 0.01; M, t = 13.42, P < 0.001; S, t = 6.98, P < 0.01, Fig. 1). Analysis of covariance revealed heterogeneity of slopes between different treatments for the effect of number of pollinator visits on outcrossing rate. Outcrossing rates tended to decrease with increases in the number of observed visits for monomorphic M and S populations, while in trimorphic populations this trend was reversed. Very little variation in outcrossing rate was observed among replicates of monomorphic L populations.

Female Fertility. — In trimorphic populations, there was no significant difference among morphs in fruit set ($\bar{x}$ = 0.96, 2 SE = 0.02; $F_{2,4}$ = 1.68, NS) or in the number of seeds per fruit ($\bar{x}$ = 92.1, 2 SE = 4.3; $F_{2,4}$ = 0.94, NS). However, seed set varied

among replicate populations ($F_{2,4}$ = 3.05, P < 0.05). In trimorphic populations, the M morph produced fewer flowers per plant than the L or S morphs which did not differ ($\bar{x}$ ± 2 SE; L, 8.5 ± 1.0; M, 7.4 ± 1.3; S, 9.0 ± 1.0; $F_{2,4}$ = 12.5, P < 0.05).

In monomorphic populations, flower production did not differ among morphs ($\bar{x}$ = 8.4, $F_{2,6}$ = 0.3, NS). Fruit set averaged 0.93 (2 SE = 0.05), with no differences among floral morphs ($F_{2,6}$ = 1.25, NS) or between monomorphic and trimorphic populations ($F_{1,4}$ = 0.7, 1.0, and 0.9 for L, M, and S morphs, respectively). The M morph produced more seeds per fruit than either the L or S morphs, which did not differ (Table 1*a*, Fig. 1). Seed set for all three morphs was lower in monomorphic than trimorphic populations, although this difference was small and nonsignificant for the M morph (Table 1*b*, Fig. 1). Analysis of covariance indicated a nearly significant trend for seed set to increase with the number of visits by *Bombus* spp. ($F_{1,7}$ = 3.9, P = 0.058).

All hand-pollinated flowers set fruit. For the L and M morphs, there were no significant differences in seed set due to pollination treatment. In the S morph, self- and intramorph pollinations produced 23% fewer seeds per fruit than intermorph pollinations (Table 2). Seed set following intermorph pollinations did not differ among morphs ($F_{2,33}$ = 1.0, P > 0.3), but was higher than seed set values from experimental populations. This may have been due to in-

TABLE 1. Seed set in experimental populations of *Eichhornia paniculata*. Nested analysis of variance of seeds per fruit in a) monomorphic populations and b) each morph in monomorphic versus trimorphic populations (Type).

a)

| | Monomorphic populations | | |
Source	df	MS	F
Morph	2	41,992.6	5.83*
Population (Morph)	6	7,206.2	20.51***
Error	305	351.3	

b)

| | | Monomorphic versus trimorphic populations | | | | | |
| Morph | | L | | M | | S | |
Source	df	MS	F	MS	F	MS	F
Type	1	52,580.9	7.8*	2,722.3	1.2	47,102.4	17.2*
Population (Type)	4	6,734.3	20.5***	2,215.6	9.1***	2,738.9	6.0***
Error	#	328.8		243.3		455.1	

*P < 0.05, *** P < 0.001.
Error degrees of freedom are 133, 134, and 135, for L, M, and S morphs, respectively.

creased pollen loads deposited by hand pollination and/or increased vigor of plants used for hand pollinations.

For populations from which pollen loads on stigmas were examined, the trimorphic population received the fewest pollinator visits (195) followed by monomorphic L (256 visits), S (352 visits) and M (400 visits) populations. Fewer pollen grains were deposited on stigmas of the L morph in the trimorphic population than stigmas of either the M or S morph ($F_{2,33}$ = 10.5, P < 0.001). Despite lower pollinator visitation to the trimorphic population, the L morph captured fewer pollen grains in the monomorphic (mon) than in the trimorphic (tri)

population ($\bar{x} \pm$ SE; mon = 129 $\pm$ 29, tri = 199 $\pm$ 60, P < 0.05). A similar, though nonsignificant trend was observed in the S morph (mon = 308 $\pm$ 39, tri = 384 $\pm$ 67, P = 0.056) while the M morph exhibited the reverse pattern (mon = 519 $\pm$ 61, tri = 409 $\pm$ 83, P = 0.064).

Male Fertility and Functional Gender. — In trimorphic populations, the percentage ($\bar{x} \pm$ 2 SE) of seeds from intermorph matings sired by the L, M, and S morphs was 23.8 $\pm$ 5.2, 32.3 $\pm$ 4.2, and 43.9 $\pm$ 6.1, respectively ($F_{2,4}$ = 10.0, P < 0.05). To correct for variation in flower production among morphs, we divided siring success in each replicate by the relative flower pro-

TABLE 2. Mean (2SE) seed set and mixed-model ANOVA for hand self-, intra-, and intermorph pollinations of the floral morphs of *Eichhornia paniculata*.

| | Morph | | |
Treatment	L	M	S
Self	105.1 (6.2)	112.5 (7.1)	85.7 (13.7)
Intra	108.8 (5.2)	117.2 (5.7)	88.8 (12.2)
Inter	111.2 (5.7)	118.6 (6.3)	120.6 (9.3)

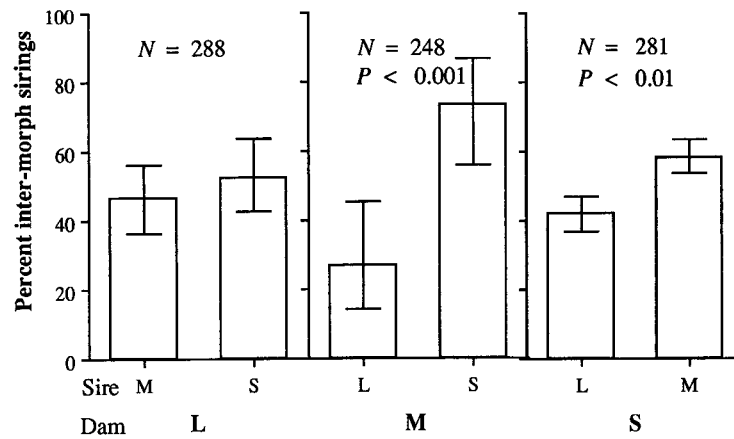| | | L | | M | | S | |
Source	df	MS	F	MS	F	MS	F
Plant	11	860.5	15.26***	1,002.6	9.28***	3,505.4	30.37***
Treatment	2	231.3	2.53	246.8	2.66	8,971.3	12.55***
Plant•treat	22	91.5	1.62	92.7	0.86	714.8	2.07*
Error	36	315.7		108.0		115.4	

*P < 0.05, *** P < 0.001.

FIG. 3.   Percent of seeds sired through intermorph mating by plants of each morph in experimental trimorphic populations of *Eichhornia paniculata*. Bars represent the range of values from the three replicates, *P*-values are from pooled *G*-statistic, N = number of seeds. Values were adjusted to reflect variation among morphs for flower number (see text). Unadjusted values gave similar probabilities. Significant heterogeneity among replicates was detected in seeds sired on the L and M morphs. In the M morph, heterogeneity reflected changes in magnitude, but not direction, of the siring asymmetry.

duction of each morph. Corrected values are 23.0 ± 6.6, 35.2 ± 5.4, and 41.7 ± 7.3, for the L, M, and S morphs, respectively, though not significantly different ($F_{2,4}$ = 5.7, P = 0.068).

Particular asymmetrical mating relationships were more striking than overall patterns of morph-specific siring ability. For instance, the L morph was three times less likely than the S morph to sire seeds produced by the M morph and also less successful than the M morph in siring seeds of the S morph (Fig. 3). Hence the L morph has relatively poor male reproductive success especially when compared to the S morph. In contrast, the M and S morphs were equally successful at siring seeds produced by the L morph.

These differences in male reproductive success did not cause large differences in gender among morphs. Variation among morphs in total seed production and rates of intermorph mating tended to obscure differences in gender due to siring success through pollen. For example, the M morph was more male than the S morph (Table 3, Eq. 2). This resulted from lower female reproductive success (due to having fewer flowers), rather than increased male reproductive success. Lower flower production by the M morph did not occur in monomorphic populations and has not been observed in nature (Husband and Barrett, 1992). Thus the differences observed in experimental trimorphic populations likely resulted from sampling error. When variation in female reproductive success and rates of intermorph mating are removed, morph has a significant effect on gender ($F_{2,4}$ = 10.1, P < 0.05) with the L morph more female and the S morph more male (Table 3).

Total outcrossing rates for the S morph

TABLE 3.   Functional gender and total outcrossing rate for each morph in experimental trimorphic populations of *Eichhornia paniculata*. See Appendix 1 for equations; st. indicates morph-specific female fertilities and rates of intermorph mating have been standardized to equal the replicate mean for all morphs. Values are means (2SE) of estimates from three replicates.

Morph	Functional gender			Total outcrossing rate		
	Eq. 1	Eq. 2	Eq. 2 st.	Eq. 3	Eq. 4	Eq. 4 st.
L	0.49 (0.02)	0.56 (0.06)	0.56 (0.04)	0.81 (0.02)	0.80 (0.05)	0.78 (0.02)
M	0.48 (0.01)	0.46 (0.04)	0.51 (0.03)	0.81 (0.004)	0.80 (0.04)	0.80 (0.01)
S	0.52 (0.02)	0.49 (0.03)	0.45 (0.03)	0.80 (0.01)	0.82 (0.06)	0.83 (0.01)

tended to be higher than seed outcrossing rates, while for the L morph this trend was reversed. This pattern resulted from the increased ability of the S morph to sire seeds through intermorph mating. However, only when variation in seed production and rates of intermorph mating are removed do the L and S morphs differ significantly in total outcrossing rate (Table 3, $F_{2,4} = 9.9$, $P <$ 0.05).

Gender values of the floral morphs did not differ greatly from 0.5, the value for an hermaphrodite that gains equal reproductive success through male and female function. We note, however, that deviation by a particular morph of ± 0.05 from the value of 0.5, means that individuals of that morph gain approximately 20% more fitness through one sex function than the other. Thus even small differences in gender values may have biological significance.

DISCUSSION

Our results support the hypothesis that reciprocal herkogamy promotes intermorph pollen transfer. Seed set and outcrossing rates were higher for all three morphs of *Eichhornia paniculata* in trimorphic than monomorphic populations. A large proportion (95%) of outcross matings in trimorphic populations were due to intermorph mating even though self and intramorph pollen is highly compatible.

The effects of reciprocal herkogamy on mating and fertility differed greatly among the floral morphs. In the L morph, outcrossing rates were only slightly higher in trimorphic than monomorphic populations, but seed set increased threefold. In contrast, seed set of the M morph was not significantly greater in trimorphic populations, whereas outcrossing rates more than doubled. In the S morph, both outcrossing rates and seed set were significantly higher in trimorphic than monomorphic populations. Thus variation among morphs in the relative placement of male and female organs caused profound differences in fertility and outcrossing in monomorphic populations. However, in trimorphic populations, outcrossing rates and seed set were high and uniform across morphs. The effects of floral polymorphism outweigh the influence of each morph's floral architecture in isolation.

Data from the L morph are consistent with the hypothesis that the selective basis for establishment of floral polymorphism in heterostylous plants could have involved improved pollen transfer rather than increased levels of outcrossing. The L morph exhibited higher pollen capture and seed set in trimorphic than monomorphic populations, apparently due to improved pollen transfer owing to reciprocal herkogamy. However, outcrossing rates of the L morph were only slightly higher in trimorphic than monomorphic populations. Comparison of the data from each morph in monomorphic populations suggests that the morphology of the L morph, with the stigma exserted beyond the anthers (approach herkogamy sensu Webb and Lloyd, 1986), reduces pollen capture but increases the proportion of pollen deposited on stigmas that comes from other plants. Presumably, outcrossing is enhanced because exserted stigmas contact pollinators before they touch anthers from the same flower. Lloyd and Webb (1992a, 1992b) argued that the ancestors of heterostylous plants were likely to have been predominantly outcrossed, by means of approach herkogamy. Under this scheme, floral heteromorphism becomes established because a variant with a shortened style, causing its anthers to extend beyond its stigma, can invade due to increased pollen export (see Barrett, 1990).

There are some difficulties, however, in interpreting our data in an evolutionary context. For our results to be relevant to selection models for the evolution of heterostyly we would need to know whether or not the initial variant was better than its ancestor at transferring pollen to the ancestral type and vice versa. Unfortunately, there is little consensus about the floral morphology of the ancestors of heterostylous species. For instance, in contrast to Lloyd and Webb (1992a, 1992b), Charlesworth and Charlesworth (1979) assume the ancestor for distylous species had styles and stamens of equal height. Even less agreement exists about the likely changes in floral morphology that have accompanied the buildup of tristyly than distyly (Yeo, 1975; Richards and Barrett, 1992). However, it seems

unlikely that the floral morphology of the ancestors closely resembled the contemporary floral morphs of tristylous species (although see Charlesworth, 1979).

The S morph also captured fewer pollen grains and had reduced seed set when in monomorphic populations. However, the number of pollen grains captured in monomorphic populations still exceeded seed set in trimorphic populations by threefold. Lower seed set in monomorphic S populations appears to result from morph-specific reductions in seed set following self- or intramorph pollination. Reduced seed set after controlled selfing of the S morph has been noted in other populations of *E. paniculata* (Barrett, 1985; Barrett et al., 1989) but intramorph pollinations were not performed in earlier experiments. Reduced seed set may be due to deleterious recessive genes linked to the dominant *S* allele (Mather and deWinton, 1941) and maintained in relatively high frequency because this allele is usually found in the heterozygous state (but see Strobeck, 1980). Reduced fertility following self- and intramorph pollination in the S morph could also be due to negative effects of homozygosity for the *S* allele itself, or weak morph-specific self-incompatibility. If the cause of the reduced fertility in the S morph is postzygotic, it would imply that inbreeding depression in *E. paniculata* may vary among morphs. Associations between genes controlling floral traits and genetic load is a component of several recent models of the evolution of inbreeding depression (Campbell, 1986; Holsinger, 1988; Uyenoyama, 1988; Charlesworth and Charlesworth, 1990), but examples of such associations are rare.

In contrast to the other morphs, the M morph did not exhibit a significant increase in seed set or pollen capture in trimorphic compared with monomorphic populations. The M morph may be more prone to self-pollination because its stigma is positioned midway between two anther levels. With this morphology, stigmas may capture more total pollen with a higher fraction originating from within a flower (Charlesworth, 1979). Pollen load data from *Pontederia* spp. (Glover and Barrett, 1986a) also demonstrate that stigmas of the M morph capture the most pollen and data on selfing rates

from monomorphic populations of the M morph in this study indicate that their ovules are more likely to be self-fertilized. The fact that the floral architecture of the M morph favors high rates of self-fertilization in monomorphic populations may explain why this particular morphology occurs rarely, if at all, in nonheterostylous plants.

While treatments differed markedly in mating patterns, outcrossing rate estimates for replicate populations of the same treatment never differed by more than 0.14. Floral morphology and morph structure therefore had much greater effects on outcrossing rate than did variation in pollinator visitation levels or fluctuations in environmental conditions. This indicates that floral morphology can exert a strong influence on the mating system, even when the environment and visitation rates vary, at least within the range encountered in these experiments. These results contrast with several other studies of mating patterns in self-compatible species in which environmental factors have been implicated as a major cause of variation in outcrossing levels (reviewed in Schemske and Lande, 1985; Barrett and Eckert, 1990).

High rates of outcrossing and intermorph mating observed in trimorphic populations could potentially result from pollination and/or postpollination phenomena. Most hypotheses concerning the function of the morphological components of heterostyly require that the floral polymorphism exert its influence on the pollination process itself. Hand pollinations demonstrated that self- and intramorph pollinations result in full seed set in the L and M morphs (and 77% seed set in the S morph) indicating that *E. paniculata* is highly self- and intramorph compatible. However, pollen types could differ in siring ability when they compete in the same style. For instance, in self-compatible, distylous *Amsinckia douglasiana,* 96% of all seeds are sired by intermorph pollen when intermorph and intramorph pollen are applied to stigmas in a mixture (Casper et al., 1988). Similarly, in *A. grandiflora,* application of mixtures of pollen from the L and S morphs resulted in 1:1 offspring morph ratios, indicating that intermorph pollen usually sired offspring (Weller and Ornduff, 1977). However, in

two other self-compatible distylous species, *A. spectabilis* (Ganders, 1975) and *Cryptantha flava* (B. B. Casper, pers. comm.), no difference in the siring ability of intermorph and intramorph pollen was detected.

In *E. paniculata*, Glover and Barrett (1986*b*) reported that while self pollen was only half as likely to sire seeds when competing with either intermorph or intramorph pollen, no difference in the competitive ability of the latter pollen types was found. Sample sizes in their study were modest and plants from our experimental populations are currently being used to investigate the siring ability of different pollen types when applied to stigmas in mixtures. Preliminary data (M. B. Cruzan and S. C. H. Barrett, unpubl. data) indicate that pollen from the same level as the stigma (legitimate pollen) has a siring advantage over both illegimate outcross pollen and self pollen while the latter two pollen types do not differ. The siring advantage of legitimate pollen, however, cannot alone account for the high levels of intermorph mating recorded in experimental trimorphic populations. Thus our results support the hypothesis that reciprocal herkogamy promotes intermorph matings by increasing the amount of legitimate pollen deposited on stigmas by insect pollinators.

Despite the use of an exotic pollinator (*Bombus* spp.) in our experiments, mean levels of outcrossing ($\bar{x} = 0.81$, 2 *SE* = 0.03) and intermorph mating ($\bar{x} = 0.77$, 2 *SE* = 0.03) were similar to values obtained in natural trimorphic populations in northeast Brazil. The outcrossing rate of the parent population (B46) in 1987 was 0.87 (M. R. Dudash and S. C. H. Barrett, unpubl. data) and outcrossing rates from 12 trimorphic populations average 0.85 (range 0.62–0.96; Barrett and Husband, 1990). The rate of intermorph mating estimated for a single trimorphic population using the style length loci as genetic markers was 0.90, SE 0.03 (Barrett et al., 1987). These results indicate that despite the artificial conditions used in our garden experiments the functioning of the polymorphism, with respect to its influence on outcrossing and intermorph mating, does not appear to have been unduly compromised. Pollinator identity may not be as critical as is often assumed for het-

erostylous systems (and see Wolfe and Barrett, 1989). Perhaps as long as pollinator tongue length is sufficient to reach the base of the perianth and contact short-level reproductive organs, enough intermorph pollen will be transferred to maintain high levels of intermorph mating.

Our investigation of mating patterns in trimorphic populations provides the first quantitative estimates of functional gender based on the use of genetic markers in a heterostylous species. Striking asymmetries in male reproductive success among floral morphs were found. Despite equal flower production, the L and S morphs differed significantly in gender due to differential ability to sire seeds through intermorph matings. Thus morph-specific gender differed from estimates using phenotypic measures based on the number of flowers and seeds produced. When genetic markers have been used, male fitness in hermaphrodite plants has frequently differed from expectations based on flower number (Stanton et al., 1986; Schoen and Clegg, 1985; Schoen and Stewart, 1986; Ennos and Dodson, 1987). Studies in which male fitness is estimated by flower number or other indirect measures (e.g., Queller, 1983; Piper and Waite, 1988) should therefore be interpreted with caution (see Snow, 1989 for a review).

Our finding that the L morph is functionally more female, and the S morph more male, is in agreement with the suggestion that, where distyly is thought to have evolved into dioecy, the L morph became female while the S morph became male (Beach and Bawa, 1980; but see Muenchow and Grebus, 1987). The floral architecture of different morphs may predispose them to greater success as male or female parents. One explanation of the low male reproductive success of the L morph may be related to Webb and Lloyd's (1986) suggestion that the female organs of approach herkogamous plants interfere with pollen removal. Another possibility is that long-level pollen from the S morph may fertilize more ovules of the M morph than short-level pollen of the L morph due to either increased transfer or siring ability. Experiments are underway to test both potential mechanisms leading to the observed differences in gender.

Our study is the first to measure how different floral morphologies affect the total outcrossing rate. Mating system studies that rely on seed outcrossing rates overlook the possibility that the pollen and ovule outcrossing rates of an individual or morph can differ. For instance, a mutation that increases pollen production could increase the proportion of selfed seeds produced by its bearer but increase the number of seeds it sires through outcrossing even more. In order to apply evolutionary models that incorporate inbreeding depression as a selective force for mating system change, it is useful to know how floral variation affects the fraction of all successful gametes found in selfed versus outcrossed seeds (Ross, 1990). In this study we found no difference in the seed outcrossing rate of morphs in trimorphic populations but small differences in total outcrossing rate due to differences in outcross pollen donation were evident.

Differences in outcross pollen donation would imply that the L morph may have lower reproductive fitness than other morphs since in most cases female fertilities apparently do not differ in natural populations (Barrett et al., 1989). However, trimorphic populations in northeast Brazil do not show reduced frequencies of the L morph as would be expected if this morph commonly had lower fitness. Rather, the S morph, which had the highest fitness in our experiments, is underrepresented in natural trimorphic populations in Brazil (Barrett et al., 1989). Moreover, Barrett et al. (1987) tested for mating asymmetries in a natural trimorphic population from northeast Brazil by comparing observed and expected morph ratios of offspring. They found no deviations from expectations based on the frequency of each morph in the population and equal siring ability of the morphs. The disparity in patterns of gender between field and garden populations may have resulted from the use of an exotic pollinator in our experiments. In particular, although this did not affect maternal reproductive parameters from those measured in the field, subtle changes in patterns of male fitness may have occurred. If this interpretation is correct, it further highlights the difficulty in obtaining reliable measures of gender in plant populations.

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APPENDIX

Hermaphroditic plants may not reproduce equally through male and female function. Lloyd (1980) suggested that the functional gender of plants be measured as the fraction of total fitness a plant gains through female function. Where the seed outcrossing rate of the

population (t) is known, the functional gender (G) of a plant can be estimated as:

$$G = \frac{f}{(1 + s)f + t\bar{f}\,\dfrac{m}{\bar{m}}}.$$

Where: f = the number of seeds produced by a plant, $s = 1 - t$, and m = the number of polliniferous flowers produced.

For a tristylous species, a morph-specific analog of the above equation that assumes outcrossed matings are disassortative and equal siring ability of flowers of each legitimate morph is:

$$G_i = \frac{f_i}{(1 + s)f_i + tf_j\,\dfrac{m_i}{m_i + m_k} + tf_k\,\dfrac{m_i}{m_i + m_j}}. \quad (1)$$

Where the subscripts i, j, and k refer to morph-specific values of each parameter.

In trimorphic populations, where morph-specific rates of intermorph mating and siring success can be estimated, realized morph-specific gender can be calculated as:

$$G_i = \frac{f_i}{(2 - d_i)f_i + f_j p_{ji} + f_k p_{ki}}. \quad (2)$$

Where d_i is the proportion of seeds of morph i sired by intermorph pollen and p_{ji} is the proportion of seeds of morph j sired by morph i.

Ross (1990) pointed out that the seed outcrossing rate (t) of a plant or morph is not sufficient for assessing the proportion of its successful gametes found in selfed vs. outcrossed seeds because pollen and ovule outcrossing rates could differ. The total outcrossing rate, T, is the proportion of all successful gametes found in outcrossed seeds. This measures combines the effects of seed and pollen outcrossing rates. If we have information only on the average seed outcrossing rate (t) of a tristylous population, and estimate male siring success as proportional to the number of flowers produced by each morph, the total outcrossing rate of a morph can be estimated as:

$$T_i = \frac{tf_i + tf_j\,\dfrac{m_i}{m_i + m_k} + tf_k\,\dfrac{m_i}{m_i + m_j}}{tf_i + tf_j\,\dfrac{m_i}{m_i + m_k} + tf_k\,\dfrac{m_i}{m_i + m_j} + 2sf_i}. \quad (3)$$

If morph-specific rates of intermorph mating and siring success are known, the total outcrossing rate can be calculated as:

$$T_i = \frac{(t_i + a_i)f_i + p_{ji}f_i + p_{ki}f_k}{(t_i + a_i)f_i + p_{ji}f_j + p_{ki}f_k + 2s_i f_i}. \quad (4)$$

Where $a_i = t_i - d_i$. Thus morph specific values for gender and total outcrossing rate based on estimated and actual male reproductive success can be compared.