Floral manipulations reveal the cause of male fitness variation in experimental populations of *Eichhornia paniculata* (Pontederiaceae)

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Summary

1. In experimental populations of self-compatible, tristylous *Eichhornia paniculata* plants of the long-styled morph (L) consistently sire fewer seeds on the mid-styled morph (M) than do plants of the short-styled morph (S). This mating asymmetry results in gender differences between the L and S morphs.

2. Observations of pollinators and floral manipulations of genetically marked plants in experimental populations were used to evaluate three potential causes of differential siring success: pollinators prefer the S morph leading to higher male success; long styles interfere with pollen pick-up by insect visitors; and pollen from long-level anthers of the S morph sires more seeds on the M morph than pollen from short-level anthers of the L morph.

3. Visitation by pollinators did not differ significantly among morphs. Excision of styles failed to increase the siring ability of the L morph on the M morph. After removal of non-mid-level anthers from the L and S morphs, no difference in siring ability was detected. Thus long-level anthers provided increased male fitness. This could occur either because pollen transfer from long-level anthers exceeds pollen transfer from short-level anthers or because long-level pollen has greater post-pollination siring success on mid-styles that does short-level pollen.

4. Differences in floral architecture may predispose morphs of heterostylous plants to differential success as male parents. Architectural constraints on male performance imposed by the placement of female and male organs within flowers may be of general importance for floral evolution.

Key-words: Floral architecture, floral manipulations, heterostyly, tristyly

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Introduction

The development and use of measures of gender in hermaphroditic plants has been an important advance in studies of floral evolution (Lloyd 1980; Ross 1990). Differences in gender represent variation among plants in the proportion of fitness gained through female *vs* male function. Relatively few attempts have been made, however, to understand the functional basis of gender variation in hermaphroditic plant populations (Schoen & Clegg 1985; Stanton *et al.* 1986, 1989, 1990; Ennos & Dodson 1987; Campbell 1989). Understanding the causes of gender variation is important because genetically based variation in gender can lead to the

evolution of separate sexes from hermaphroditism (Charnov 1982; Lloyd 1982; Charlesworth 1989) and knowledge of the factors causing variation in female and male reproductive success furthers understanding of how selection acts on floral traits. Here we take an experimental approach using floral manipulations and genetic markers to evaluate the mechanism of gender variation among floral morphs of a heterostylous species.

In a previous study using experimental populations of self-compatible tristylous *Eichhornia paniculata* (Spreng.) Solms. (Pontederiaceae), morph-specific gender differences arose because plants of the short-styled morph (S) were more successful at siring seeds on the mid-styled morph (M) than plants of the long-styled morph (L) (Kohn & Barrett 1992). The S morph gained more than half of its reproductive success through male function while the reverse was true for the L morph. Differences among morphs in

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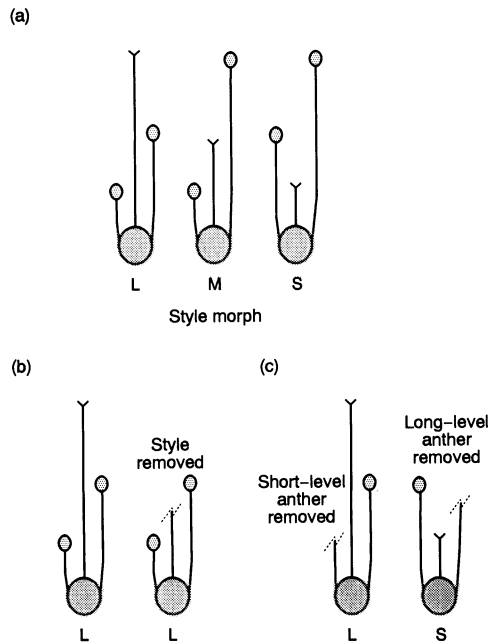
Fig. 1. Treatments used in experimental populations of *Eichhornia paniculata:* (a) unmanipulated tristylous floral morphology; (b) styles removed from half of the plants of the L morph; (c) short-level anthers removed from plants of the L morph, long-level anthers removed from plants of the S morph.

floral architecture apparently lead to differences in male reproductive success. The purpose of this study is to evaluate the mechanism(s) of the siring advantage of the S morph to help understand what characteristics of heterostylous flowers are likely to lead to increased male reproductive success.

At least three mechanisms could account for the greater siring success of the S morph:

1. *Pollinator preference.* Pollinators could prefer to visit the S morph rather than the L morph because flowers of the S morph present more exserted anthers from which pollinators may more effectively harvest pollen. Increased visitation could lead to increased siring success. Detailed observations were made to see if pollinators foraged preferentially among morphs.

2. *Pollen–stigma interference.* The male and female organs within a flower may interfere with each other's function (Lloyd & Webb 1986; Webb & Lloyd 1986). One way in which pollen–stigma interference could operate is if the long style of the L morph interferes with pollen pick-up by insects causing reduced male success. We tested this hypothesis by measuring the ability of plants of the L morph with their styles intact or excised to sire seeds on the M morph (Fig. 1).

3. *Differential pollen success.* Pollen from any other anther level can successfully sire seed on any style morph of *E. paniculata* (Kohn & Barrett 1992). While both L and S morphs bear mid-level anthers, the S morph may gain a siring advantage over the L

morph if long-level pollen more frequently sires seeds on the M morph than does short-level pollen. The differential pollen success hypothesis was tested by removing the long-level anthers of the S morph and the short-level anthers of the L morph and comparing the ability of plants bearing these altered flowers to sire seeds on the M morph (Fig. 1).

Materials and methods

The experiments were performed in southern Ontario using *Bombus* spp. as the principal pollinators. Plants used in this study were derived from population B46 in Ceára, north-east Brazil where common pollinators include long-tongued Anthophorid bees of the genera *Florilegus* and *Ancyloscelis* (Husband & Barrett 1992). Kohn & Barrett (1992) found that rates of outcrossing and disassortative mating in experimental and natural populations of *E. paniculata* were similar. In this study we investigated the causes of morph-specific differences in male success which have been observed using genetically marked experimental populations visited by bumble-bee pollinators. Our manipulations were aimed at ascertaining the cause of male fertility variation under these same conditions.

All plants used in this study were homozygous at the triallelic locus *AAT-3*. In experimental populations, plants of each morph were homozygous for different alleles at *AAT-3* allowing the morph of the paternal parent to be inferred from alleles at *AAT-3* in seed. The association of morphs and electrophoretic alleles was altered among replicates of the same treatment. Each plant had a single inflorescence and the total number of flowers per morph was equal, although some variation in flower number among plants within morphs remained. Populations were exposed to pollinators for a single day (flowers last 6 h in this species) in August and September 1990, and mature fruits were removed 11–12 days later. Details of the origin of plants and electrophoretic procedures used in this study can be found in Kohn & Barrett (1992).

Three types of experimental populations (treatments) were used (Fig. 1). In each population, equal numbers of plants of all three morphs were used and plants were placed at random approximately 30 cm apart in 6 × 6 (unmanipulated and anther removal treatments) or 6 × 8 (style removal treatment) arrays. The treatments were:

1. Unmanipulated. Populations were constructed using 12 plants of each morph. This treatment was replicated three times and was identical to experiments conducted in 1989 (Kohn & Barrett 1992) with the exception that in the earlier experiment, flower number per morph was not controlled.

2. Style removal. Styles were removed below the mid-level anthers using fine forceps during the hour prior to the onset of anther dehiscence on half of the

Table 1. The number of (a) pollinator visits to plants and (b) flowers probed per visit to style morphs of *Eichhornia paniculata* in experimental populations. Fractions for the L morph in the style removal treatment represent plants with styles excised/intact

(a) Pollinator visits to plants

Treatment†	L	M	S	G_p (df)	G_h (df)
UN	200	196	216	1·1 (2)	3·7 (4)
SR	99/95	200	213	1·0 (3)	4·0 (6)
AR	84	104	115	5·0 (2)	0·1 (2)

(b) Flowers probed per visit

	Mean ± SE			Morph			Replicate			Interaction		
Treatment	L	M	S	MS	df	F	MS	df	F	MS	df	F
UN	4·4 ± 0·2	4·5 ± 0·2	4·6 ± 0·2	0·7	2	1·5	6·0	2	12·8*	0·5	4	1·2
SR	2·9 ± 0·2/3·2 ± 0·2	3·3 ± 0·2	3·5 ± 0·2	0·9	3	3·2	0·1	3	0·5	0·3	6	0·9
AR	2·9 ± 0·2	3·5 ± 0·2	3·0 ± 0·2	0·5	2	10·7	1·2	1	22·5*	0·05	2	0·9

†Treatments: UN, unmanipulated; SR, style removal; AR, anther removal. Pollinator visits analysed by *G*-test ($G_p = G$ for pooled data; $G_h = G$ for heterogeneity among replicates), flowers probed per visit were analysed by ANOVA.
*$P < 0.05$.

plants of the L morph in this treatment. All plants used were homozygous at an additional diallelic locus, *PGI-2*, and plants of the L morph with styles excised *vs* intact were homozygous for alternative alleles. Thus for seeds sired by the L morph, we could determine whether a manipulated or unmanipulated plant was the father. To increase the probability of detecting a difference in siring between the two types of plants of the L morph, population size was increased to 16 plants of each morph and four replicates of this treatment were performed.

3. Anther removal. Long-level anthers were removed from all plants of the S morph and short-level anthers were removed from all plants of the L morph using fine forceps during the hour prior to the onset of anther dehiscence. Hence only mid-level anthers remained on flowers of the L and S morphs. Filaments of long level anthers were left intact so that the body position of insects visiting flowers of the S morph would be altered as little as possible. Twelve plants of each morph were used and two replicates of this treatment were completed.

In all experimental populations, pollinator foraging bouts were observed for the first 3 h after visitation began. The number of plants of each morph visited, and the number of flowers probed per visit were recorded. Occasionally more than one pollinator visited the population simultaneously but only the foraging activity of the first-arriving pollinator could be followed. The effects of morph and replicate on the number of inflorescences of each morph visited were analysed using replicated *G*-tests. Analysis of variance was used to evaluate the effects of morph (fixed effect), replicate (random effect) and their interaction (random effect) on the square root of the number of flowers visited per inflorescence. Square-root transformation successfully removed heteroskedasticity.

Seeds were counted from two fruits from each plant in each experimental population in order to test for morph-specific variation in female reproductive success. Morph, treatment and their interaction were entered as fixed effects while replicates and the interaction of morphs with replicates were random effects in the analysis of variance of average seed set per plant. Appropriate *F*-ratios were determined using the expected mean squares produced by JMP version 2·04b (SAS Institute 1989).

Five seeds from each of two fruits from each plant of the M morph were assayed electrophoretically to determine the morph of the sire. Data on the number of seeds sired by the L and S morphs on the M morph were then analysed using replicated *G*-tests. The association between treatments and the morph that sired seeds on the M morph was evaluated using log-linear analysis (JMP version 2·04b, SAS Institute 1989).

Results

The number of inflorescences visited and the number of flowers probed per visit did not differ among morphs in any of the three treatments (Table 1). For the style removal treatment, visitation to plants of the L morph with styles intact and excised did not differ (visits, $G_{pooled,1} = 0.08$, NS; flowers probed, $F_{1,3} = 1.24$, NS). Only in the anther removal treatment did pollinator discrimination among morphs approach statistical significance ($0.1 > P > 0.05$ for both inflorescences visited and flowers probed per visit).

After excluding plants of the L morph with their styles removed, treatments differed in the number of seeds per fruit ($\bar{x} \pm$ SE; unmanipulated, 88·2 ± 2·1; style removal, 96·3 ± 1·5; anther removal, 80·2 ± 3·1; $F_{2,6} = 8.4$, $P < 0.05$). Increased seed set in the style removal treatment may result from larger

Table 2. The number of seeds on the M morph sired by the L and S morphs in experimental populations of *Eichhornia paniculata*. For style removal treatment, fractions represent the number of seeds sired by plants of L morph with styles removed/intact

Treatment	Rep	Seeds sired on the M morph			
		L	S	G	
UN	1	26	49	7·1**	
	2	34	52	3·8	
	3	16	25	2·0	
	Total	76	126	12·5***	(pooled)
				0·4	(heterogeneity)
SR	1	15/27	85	14·8***	
	2	28/32	71	0·9	
	3	26/28	93	10·4**	
	4	31/20	90	10·9***	
	Total	100/107	339	32·2***	(pooled)
				4·8	(heterogeneity)
AR	1	43	47	0·2	
	2	29	32	0·1	
	Total	72	79	0·3	(pooled)
				0·0	(heterogeneity)

Treatments as in Table 1. Degrees of freedom equal 1 for within-replicate tests, 1 for pooled tests, and the number of replicates minus one for heterogeneity tests.
$**P < 0.01$; $***P < 0.001$.

population size or a higher ratio of pollen to stigmas. Lower seed set in the anther removal treatment probably reflects the removal of one-third of the anthers in these populations. The morph by replicate interaction was also signficant ($F_{12,313} = 2·0$, $P < 0·05$) indicating variation in seed production among morphs across replicates of the same treatment.

In the unmanipulated treatment, the S morph sired 1·66 times as many seeds on the M morph as did the L morph (Table 2). This result was consistent in direction, though lower in magnitude, to results obtained previously (Kohn & Barrett 1992). In the style removal treatment, plants of the L morph with excised and intact styles did not differ in siring ability on plants of the M morph (Table 2; $G_{pooled,1} = 0·24$, NS; $G_{het,3} = 3·5$, NS) and the relative siring advantage of the S morph (1·64) was virtually identical to that found in the unmanipulated controls ($\chi^2_1 = 0·005$, $P = 0·94$). In the anther removal treatment, however, there was no significant difference in the ability of the L and S morphs to sire seeds of the M morph (Table 2). The ability of the L and S morphs to sire seeds on the M morph was significantly different between the anther removal and unmanipulated treatments by a one-tailed test of the hypothesis that the treatment would reduce the advantage of the S morph ($\chi^2_1 = 3·6$, $P = 0·029$). Siring of seeds of the M morph by the L and S morphs differed significantly between anther removal and style removal treatments ($\chi^2_1 = 4·7$, $P = 0·03$, two-tailed). If style removal and unmanipulated treatments are lumped, then the effect of anther removal is significant by the more conservative two-tailed test ($\chi^2_1 = 5·1$, $P = 0·02$).

Discussion

Preferential visitation by insect pollinators to floral morphs of *E. paniculata* is unlikely to account for the differences in siring ability observed in experimental populations. No significant differences were found in either the number of inflorescences visited or the number of flowers probed per inflorescence. Only in the anther removal treatment did pollinator discrimination among morphs approach statistical significance, and this was the treatment under which the siring success of the L and S morphs did not differ. We also found no evidence to support the hypothesis that the pistil of the L morph interferes with male function. Removal of the style had no effect on the siring success of the L morph on the M morph. When the L and S morphs had non-mid-level anthers removed, however, their ability to sire seeds on the M morph did not differ signficantly. Thus differential success of pollen from long- vs short-level anthers best explains the siring advantage of the S morph.

Three types of mechanisms, acting either alone or in concert, could account for greater success of long- vs short-level pollen on styles of the M morph. First, in both natural and experimental populations of *E. paniculata*, long-level anthers dehisce before mid-level anthers which, in turn, dehisce earlier than short-level anthers. Observations of anthesis indicate that up to an hour may elapse between long- and short-level anther dehiscence and the length of time separating dehiscence among anther levels appears to vary with temperature and humidity. Similar dehiscence patterns are also evident in the related tristylous *Pontederia cordata* (L. D. Harder & S. C.

H. Barrett, unpublished data). Differences in dehiscence time could result in long-level pollen gaining precedence over short-level pollen if transfer of long-level pollen begins before short-level anthers dehisce. First-arriving pollen has been shown to have a siring advantage in *E. paniculata* (Graham & Barrett 1990) and also in *Ipomoea purpurea* (Epperson & Clegg 1987). The contribution of staggered anther dehiscence to the siring advantage of the S morph could be evaluated by exposing populations to pollinators after all three anther levels were fully dehisced and comparing the results to mating patterns from populations where pollinators were available from the onset of anthesis.

Second, transfer of pollen from long-level anthers to mid-level stigmas may be more frequent than transfer from short-level anthers. The exsertion of long-level anthers beyond the flair in the perianth tube could promote transfer through improved pollinator contact. Differential transfer could not be due to differential pollen production since short-level anthers produce more grains than do long-level anthers (Barrett 1985). *Eichhornia paniculata* lacks the strong pollen size heteromorphism commonly found in self-incompatible heterostylous species making measurement of deposition of alternative pollen types difficult. However, while pollen size distributions from adjacent anther levels overlap broadly, there is little overlap in the sizes of pollen from long- and short-level anthers. Thus it may be possible to measure the relative rates of deposition from long- and short-level anthers if mid-level anthers are removed.

Third, differences in the ability of plants of the L and S morphs to sire seeds on the M morph could result from greater post-pollination siring success of long-level pollen. Experiments with mixtures of genetically marked pollen indicate that long-level pollen has a post-pollination siring advantage over short-level pollen on styles of the M morph (Barrett, Kohn & Cruzan 1992; Cruzan & Barrett 1992). By removing mid-level anthers from plants of the L and S morphs and estimating both pollen deposition and success in siring seeds of the M morph it may be possible to determine the relative contributions of pollination and post-pollination processes. Post-pollination processes, however, are unlikely to be the sole determinant of mating patterns observed in experimental populations of *E. paniculata*. In trimorphic populations, plants of the M morph sire more seeds on the S morph than do plants of the L morph (Kohn & Barrett 1992). While the mechanism of this second mating asymmetry has not been investigated, it is unlikely to be due to post-pollination processes because on styles of the S morph, mid-level pollen, which the L morph produces, has a siring advantage over long-level pollen, which the M morph produces. This implies that a male advantage may accrue through pollen transfer or precedence to morphs producing long-level anthers.

In tristylous species, each morph has two levels of anthers that occupy the positions not occupied by the stigma (Fig. 1). One morph must, therefore, lack long-level anthers. If these anthers are more proficient at transferring pollen, or dehisce earlier because the microclimate is less humid around exserted anthers than those occurring deeper within the floral tube, then the morph lacking them may have lower male reproductive success, at least in self-compatible species. Thus architectural constraints imposed by heterostylous floral morphology can affect the performance of morphs as male parents and lead to differences in functional gender. Gender variation can promote the evolution of differential allocation to male and female reproductive success among morphs in heterostylous species (Casper & Charnov 1982; Charlesworth 1989; Casper 1992). Discovering the mechanisms by which gender differences arise may help evaluate selective forces that can lead to gender specialization and the evolution of separate sexes.

Architectural constraints on male success of flowers may also occur in monomorphic species (Lloyd & Webb 1986; Webb & Lloyd 1986; Campbell 1989). For instance, in species with stigmas exserted beyond anthers (approach herkogamy), an individual with increased stamen length might achieve greater outcross pollen donation because its anthers would be held at a level closer to that of the stigmas of other plants. However, such an individual might pay a cost in terms of increased levels of self-pollination due to reduced stigma–anther separation within its own flowers. In self-incompatible species, increased self-pollen deposition may potentially reduce seed set through stigma clogging, while in self-compatible species increased self-pollination could increase the rate of self-fertilization and reduce seed fitness. Finally, increased stamen length might not increase outcross pollen donation if interference of anther function by the stigma increases with decreasing stigma–anther separation (Webb & Lloyd 1986). Failure to detect interference in this study does not mean that interference may not at times be a potent force affecting floral evolution. It is possible that the lack of demonstrable interference resulted from the evolutionary adjustment of stigma–anther separation so as to minimize it. In fact, one suggested selective force promoting the evolution of heterostyly is the reduction of stigma–anther interference (Webb & Lloyd 1986; Lloyd & Webb 1992).

Previous studies of the way in which architectural variation in floral traits affect female and male reproductive success of hermaphroditic plants have largely used correlative approaches to explore relationships between morphological variation and components of reproductive success (e.g. Campbell 1989; Stanton *et al.* 1990; Campbell *et al.* 1991).

While these approaches are useful for generating hypotheses about the causes of reproductive variation among plants, they cannot demonstrate that the traits under study cause the variation. Unmeasured correlated characters may be the actual cause of reproductive variation. In addition, the mechanism by which character variation leads to reproductive differences cannot be explicitly tested by these methods. The use of floral manipulations and genetically marked plants provides a powerful tool with which to evaluate competing hypotheses about the mechanisms causing differences in male fitness among individuals in populations of hermaphroditic plants. These methods allowed us to reject two of the three hypothesized causes of variation in male reproductive success. Floral manipulations are subject to the caveat that they may affect visitation rates or the way in which flowers are handled by pollinators. Thus they may not test the direct effects of the structural alterations. However, when used in conjunction with pollinator observations, they can help discern the mechanisms by which morphological variation leads to fitness differences. These techniques are not limited to heterostylous systems and should prove useful in evaluating structural constraints on floral function in monomorphic species as well.

Acknowledgements

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