Modification of flower architecture during early stages in the evolution of self-fertilization

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INTRODUCTION

The evolutionary transition from outcrossing to predominant self-fertilization in flowering plants is commonly associated with modifications to a range of floral traits (table 1 in Ornduff, 1969). Compared with their outcrossing relatives, selfing species generally have smaller less-conspicuous flowers, reduced physical separation between anthers and stigmas – hereafter herkogamy – and lower pollen:ovule ratios [e.g. Leavenworthia (Lloyd, 1965), Mimulus (Ritland and Ritland, 1989) and Collinsia (Armbruster et al., 2002)]. However, determining the sequence of floral modifications that culminate in the ‘selfing syndrome’ has remained a challenge for evolutionary biologists. Although the evolution of selfing is the most frequent mating-system transition in plants (Stebbins, 1974), it is not clear which changes initiate increased rates of selfing and which accumulate after a species has become largely selfing. Plant species that display inter-specific variation in mating system provide opportunities to investigate early stages in the transition towards self-fertilization.

In self-compatible species, herkogamy has long been considered a major determinant of mating patterns in plant populations (Darwin, 1862; Müller, 1883; Webb and Lloyd, 1986). Reduced herkogamy correlates positively with fruit set in both the presence (Rick et al., 1978) and absence of pollinators (Ennos, 1981; Carr and Fenster, 1994; Elle and Hare, 2002), self-pollen deposition (Thomson and Stratton, 1985) and marker-based estimates of selfing (Barrett and Shore, 1987; Holtsford and Ellstrand, 1992; Motten and Antonovics, 1992; Brunet and Eckert, 1998; Takebayashi et al., 2006). Furthermore, studies that have statistically controlled for the effect of other traits potentially associated with herkogamy, such as flower size, have clearly shown that herkogamy can be a primary determinant of autonomous seed set and rates of self-fertilization (Brunet and Eckert, 1998; Elle and Hare, 2002), although some exceptions do occur (Medrano et al., 2005).

Variation in herkogamy is heritable in diverse animal-pollinated species (Ennos, 1981; Shore and Barrett, 1990; Herlihy and Eckert, 2007; Kulbaba and Worley, 2008) and...
TABLE 1. Phenotypic (above diagonal) and maternal family (below diagonal) Spearman-rank correlations between flower number, plant size, days to flowering and flower morphology in Eichhornia paniculata from north-east Brazil (trimorphic populations only)

<table>
<thead>
<tr>
<th></th>
<th>Days to 1st flower</th>
<th>Plant size (PC1)</th>
<th>Flower no.</th>
<th>Perianth size</th>
<th>Nectar-guide size</th>
<th>Stigma–anther separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days to 1st flower</td>
<td>—</td>
<td>0.034 (0.398)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Plant size (PC1)</td>
<td>0.189 (0.028)</td>
<td>—</td>
<td>0.211 (&lt;0.0001)</td>
<td>0.009 (0.828)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Flower no.</td>
<td>—</td>
<td>—</td>
<td>0.551 (&lt;0.0001)</td>
<td>0.156 (&lt;0.0001)</td>
<td>0.209 (&lt;0.0001)</td>
<td>0.111 (0.006)</td>
</tr>
<tr>
<td>Perianth size</td>
<td>—</td>
<td>0.060 (0.496)</td>
<td>0.539 (&lt;0.0001)</td>
<td>—</td>
<td>0.206 (0.017)</td>
<td>—</td>
</tr>
<tr>
<td>Nectar-guide size</td>
<td>0.027 (0.754)</td>
<td>0.180 (0.037)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Stigma–anther separation</td>
<td>0.210 (0.015)</td>
<td>0.096 (0.271)</td>
<td>0.522 (&lt;0.0001)</td>
<td>0.251 (0.003)</td>
<td>0.171 (&lt;0.0001)</td>
<td>0.145 (0.094)</td>
</tr>
</tbody>
</table>

Nominal statistical significance is shown in parentheses. Bold face indicates significance after a sequential Bonferroni correction. PC1 = first eigenvectors of the principal component analysis of the correlation matrix of vegetative (plant size) traits. Sample size for phenotypic correlations n = 607–620 plants, mean 3–61 flowers per individual, range 1–18; genetic correlations n = 132–134 families, mean 4–62 plants per family, range 1–12.

has been shown to respond to artificial selection (Chang and Rausher, 1998). Less is known about the role of environmental factors in modifying the expression of herkogamy (but see Brock and Weining, 2007) and to what extent this trait exhibits phenotypic plasticity – the ability of a genotype to express alternative phenotypes under different environmental conditions (Bradshaw, 1965). Plasticity in herkogamy could allow genotypes to adopt different mating strategies depending on local environmental conditions. For example, in plants where outcrossing is generally favoured, stressful conditions, e.g. due to seasonal droughts or limited pollinator service, may favour self-pollination as a mechanism of reproductive assurance (Stebbins, 1957; Levin, 1972; Moeller and Geber, 2005). According to this scenario phenotypic plasticity of herkogamy expression could be adaptive and represent an evolutionary response to heterogeneous environments (Via et al., 1995).

The annual tristylous aquatic plant Eichhornia paniculata provides an opportunity to investigate modifications to flower architecture during the early stages in the evolution of self-fertilization. In the central part of its distribution in north-east Brazil, populations display a wide range of mating patterns (Barrett and Husband, 1990). Tristylous populations contain long-, mid- and short-styled morphs (hereafter L-, M- and S-morphs) and are largely outcrossing, whereas dimorphic (L- and M-morphs) and monomorphic (M-morph only) populations exhibit variation in selfing rates, depending on the presence of self-pollinating variants of the M-morph with reduced herkogamy (Barrett et al., 1989). As a result, the selfing rate of populations is correlated with among-population variation in the degree of herkogamy in the M-morph (fig. 3b in Barrett and Husband, 1990). In Brazilian populations of E. paniculata, reduction in herkogamy in the M-morph is largely achieved through elongation of a single short-level stamen (Séburn et al., 1990; Richards and Barrett, 1992). Predominantly selfing populations of E. paniculata on the Caribbean Islands of Jamaica and Cuba are composed primarily of the M-morph and have much smaller and less-showy flowers, lower pollen:ovule ratios and up to three elongated stamens in the mid-level position (Barrett, 1985; Morgan and Barrett, 1989, S. C. H. Barrett, unpubl. res.). These highly autogamous variants are described as semi-homostylos (Ornduff, 1972). Hence, E. paniculata displays the full spectrum of floral modifications associated with the evolution of the selfing syndrome.

In addition to significant population-level differentiation in floral traits, plants of E. paniculata also display remarkable patterns of within-individual variation. The variation can be expressed in several ways, including developmental instability in perianth shape resulting from differences in tepal number, and variation in the position of stamens within a flower as a result of differences in filament elongation (Barrett, 1985, 1996; Richard and Barrett, 1992). Developmental instability in stamen position is of particular functional significance because it can influence the facility for autonomous self-pollination of flowers and hence mating patterns. Plants in dimorphic and monomorphic populations commonly produce a mixture of flowers, some with well-developed herkogamy and others in which herkogamy is weakly developed or absent resulting in autonomous self-pollination (Séburn et al., 1990; Barrett and Harder, 1992). These two flower types have been referred to as unmodified and modified flowers, respectively, and are particularly characteristic of plants of the M-morph. The occurrence in E. paniculata of developmental instability in herkogamy provides an opportunity to investigate the genetic and environmental control of within-individual variation in a trait of obvious functional significance to mating patterns.

Here, using comparisons of populations grown under uniform glasshouse conditions and experimental manipulation of growing conditions, the morphological changes associated with the evolution of selfing in E. paniculata are investigated, focusing in particular on populations from north-east Brazil because in this region the early stages in the transition from outcrossing to selfing are evident (Barrett et al., 1989). Therefore plants have not been included from other geographic areas (e.g. Caribbean and Central America) where populations are small flowered and highly selfing (Barrett, 1985; Barrett and Husband, 1990, S. C. H. Barrett, unpubl. res.). In the present study, particular attention is paid to variation in herkogamy, because previous work has demonstrated that it is functionally associated with variation in the mating system of E. paniculata. The following specific questions were addressed in this study: (a) Is within- and among-population variation in herkogamy associated with other floral and vegetative traits, especially flower size? (b) To what extent is variation in herkogamy limited to the M-morph, especially in segregating families containing self-pollinating variants and individuals of the L-morph? (c) What are the relative roles of genetic and environmental variation in the expression of herkogamy,
and how phenotypically plastic is this trait? (d) Based on measurements of within-plant variation, what are the patterns of developmental instability in herkogamy among individuals? It is proposed that the regulation of herkogamy may provide populations of *E. paniculata* with the potential for dynamic control of mating in the face of unpredictable environmental conditions.

**MATERIALS AND METHODS**

**Study populations and glasshouse culture**

Thirty-one Brazilian populations of *Eichhornia paniculata* (Spreng.) Solms. (Pontederiaceae), collected as open-pollinated progenies from maternal parents in May–June 2005, were investigated. The localities and morph structure of populations [i.e. whether populations are trimorphic (L-, M-, S-morph), dimorphic (L-, M-morph) or monomorphic (M-morph only)] are provided in Table S1 in Supplementary Data, available online). The sample included 18 trimorphic, 11 dimorphic and two monomorphic populations. In the glasshouse, seeds from up to ten maternal families per population were sown on 11 August 2006 onto wet soil. Pots were randomly placed in plastic water-filled trays (30 pots per tray) and maintained at approx. 34°C to stimulate germination. After germinated seedlings reached the two-to-three-leaf stage, pots were transferred to a cooler glasshouse (25°C), and each tray was fertilized with 150 mL of a 300 ppm solution of 20:20:20 fertilizer (Plant Products Co. Ltd, Brampton, Ontario, Canada). Beginning on 21 September, up to 12 seedlings from each maternal family were transplanted into 5.7-cm pots and randomly assigned to water-filled trays on a glasshouse bench. Plants received sporadic fungicide treatment (No-Damp fungicide; Plant Products Co.) and a weekly dose of 150 mL of liquid fertilizer. Fertilizer concentration was doubled once flowering began to maintain plant growth.

**Measurements of floral and vegetative traits**

Date to first flower and floral morph identity (L-, M- or S-morph) for each plant were recorded between 27 October 2006 and 8 January 2007, when the vast majority of plants had flowered. For the first two inflorescences produced by each plant, four randomly chosen flowers (two per inflorescence) were measured for the following characters: (a) vertical and horizontal width of the perianth; (b) width and length of one of the two nectar guides; and (c) stigma–anther separation (herkogamy), measured from the stigma surface to the midpoint of the nearest anther, which corresponded to one of the short-level anthers in the M-morph, and a mid-level anther in the L-morph (see Fig. 1). In addition, the following were measured: (d) the size of the leaf (bract) subtending the first inflorescence (width, length to petiole, and length to base); (e) plant height from the soil surface; and (f) flower number per inflorescence. All measurements were made with digital calipers to the nearest 0.01 mm, except plant height which was measured with a ruler to the nearest centimetre.

**Correlations among traits.** To determine the extent to which floral traits co-varied among themselves, as well as with plant size and flowering time, phenotypic and genetic (maternal family) correlations were estimated using Spearman rank-correlations. Genetic correlations were approximated by calculating correlations of family means (Falconer and Mackay, 1996). Because only the correlation among traits within populations in which selfing adaptations arise was of interest, this analysis was restricted to trimorphic populations only. Perianth and nectar guide size were calculated as the product of length × breadth. This measurement of perianth size has been used in previous studies of *E. paniculata* and correlates well with floral dry weight (Worley and Barrett, 2001). The four measured vegetative traits were summarized using principal component analysis of the correlation matrix, and the first principal component – which explained 67-12% of the variance and was positively correlated with all vegetative traits (data not shown) – was used for this analysis. All analyses were conducted using the statistical program R ver. 2.6.2 (R-Development Core Team, 2008).

**Comparisons among floral morphs.** Floral traits among morphs were compared using non-parametric Wilcoxon signed-rank tests. Non-parametric tests were chosen for these analyses because the bimodal distribution of herkogamy in some individuals of the M-morph (see below) could not be easily transformed to normality. Because calculated correlations used combined data across populations the estimates include the contribution of both genetic variation within populations and differentiation among populations (Arnbuster, 1991). Both trimorphic and dimorphic populations were included in this analysis, but they were analysed separately.

**Patterns of herkogamy variation in the floral morphs.** Previous work (Seburn et al., 1990; Barrett and Harder, 1992) and preliminary observations of the populations suggested that in some plants of the M-morph the distance separating stigmas and anthers was bimodally distributed. To characterize this pattern, a mixed-distribution analysis was conducted using
the module *mixdist* (MacDonald and Du, 2008). The module *mixdist* uses maximum likelihood to estimate the parameters of a mixed distribution formed by the combination of *k* unimodal distributions. For the mixed-distribution analysis, a combination of two normal distributions was fitted to the observed frequency of stigma–anther distance and this was compared with the expected distributions using a *χ*²-test. The maximum likelihood estimates of the mean, variance and relative frequency (mixing proportion) of the predicted combination of the two normal curves were also obtained.

To determine if herkogamy variation was largely confined to the M-morph, a correlation analysis was used to investigate the extent to which stigma–anther separation values among maternal families of the M-morph segregating both L- and M-individuals (plants of genotype *ssMm*; see Barrett *et al.*, 1989) from trimorphic and dimorphic populations were associated. If modification of herkogamy is not morph specific, a correlation between values for stigma–anther distances in plants of both the L- and M-morph from the same maternal family would be expected. In contrast, if the genetic modifications to herkogamy are morph-specific there should be no correlation between stigma–anther separation in segregating families. Stigma–anther separation for 2384 flowers from 685 individuals from 111 segregating families was measured. The mean stigma–anther distance was calculated for each morph within each family and these values used for calculating Spearman rank-order correlations.

To analyse the bimodal expression of herkogamy across different levels of organization (population morph structure, population, family and individual plant) stigma–anther distance was transformed into a binary trait. Flowers were classified into either modified (herkogamy ≥ 2 mm) or unmodified (herkogamy < 2 mm). The threshold of 2 mm was chosen *a priori* following a preliminary examination of flowers, which indicated that this value corresponded to the lowest point between the two modes in the frequency distribution of stigma–anther distance (Fig. 2). A mixed-effects model with a binomial error and a logit link function was then fitted to the transformed data using the module *lme4* (Pinheiro and Bates, 2000). The use of mixed effects models allowed the structure inherent to the data set to be incorporated. The unit of observation was the flower, which was nested within plant, family and population (random effects). Population morph structure was treated as a fixed effect and flower size (perianth area) as a covariate. For the response variable (stigma–anther distance), modified flowers were coded as (1) and unmodified flowers (0). Statistical significance of individual terms was assessed via likelihood ratio tests (LRT) of the full model versus a model excluding the term of interest.

### Phenotypic plasticity in stigma–anther separation

To determine the extent to which herkogamy is influenced by growing conditions during development, flower and vegetative traits of a sample of genotypes were compared in two contrasting environments. Only individuals of the M-morph were used in the experiment because this morph displays considerable variation in herkogamy (see Results). A sample of ten genotypes representing the range of variation in herkogamy observed in the 31 populations sampled above was chosen. The genotypes originated from one trimorphic (two genotypes), four dimorphic (six genotypes) and one monomorphic (two genotypes) populations (for details see Table S2 in the

![Fig. 2. Frequency distribution of stigma–anther separation (top) and perianth length (bottom) for long- (L), mid- (M) and short-styled flowers (S) from 31 populations of *Eichhornia paniculata* from north-east Brazil grown under uniform glasshouse conditions. *n* = 1374, 2365 and 378 flowers from *n* = 400, 685 and 107 plants of the long-, mid- and short-styled morph, respectively.](image-url)
Supplementary Data, available online). To encourage clone production via axillary shoots for each of these genotypes, plants were transplanted on 23 May 2007 to 10-2-cm pots with a soil mix of 1:1 soil:sand in 5-L plastic buckets. Water levels were kept above the soil level and plants were fertilized weekly with 250 mL of 900 ppm 20:20:20 fertilizer. On 8 August axillary shoots produced by all genotypes were excised from maternal plants and transplanted to separate pots. Population origin and the number of ramets (clones) per genotype (genet) are given in Table S2.

To impose different growth conditions to ramets of the same genotype, the cloned axillary shoots from each genotype were randomly assigned to each of two treatments that differed in pot size, water level and nutrient availability. The two contrasting growth conditions are referred to as ‘low stress’ and ‘high stress’ for convenience. Ramets in the low-stress environment were grown in 10-2-cm pots, the water level was maintained above the soil, and plants were fertilized weekly with 250 mL of a 900 ppm solution of 20:20:20 liquid fertilizer. In contrast, ramets in the high-stress treatment were grown in smaller pots (7.6 cm), water levels were much reduced and plants were fertilized weekly with 50 mL of fertilizer. At the start of the experimental treatments, leaf size and height of the main stem were measured, and a single measure of plant size obtained using principal component analysis of the correlation matrix as described above. There was no significant difference among ramets transplanted to the two treatments (t = -0.606, d.f. = 69:34, P = 0.546), indicating that the sample of ramets in each treatment was of equivalent size. Starting on 28 August, traits were measured on ramets in the two growth treatments. For the first two inflorescences produced by each plant, leaf size and leaf height were measured as described above. For the first five flowers produced by each ramet, perianth and nectar guide length and width were measured and for 25 flowers per inflorescence (up to 55 per ramet) stigma–anther separation was measured.

Data from this experiment were analysed using logistic regression (Barrett and Harder, 1992). For each ramet, the number of modified (stigma–anther distance <2 mm) and unmodified flowers was counted and pooled across inflorescences within genets. The response variable for this analysis was a matrix of the number of modified and unmodified flowers for each plant (n = 72 ramets; n = 3548 flowers). Perianth size was calculated as the mean area of the five flowers measured per ramet. Because the ten genotypes included in this experiment were chosen to represent the range of herkogamy variation, genotype was treated as a fixed effect. Treatment was also treated as a fixed effect and mean perianth size was used as a covariate. The model was fitted using the module glm with a binomial error and a logit link function and statistical significance was assessed via LRT of the full model versus a model excluding the term of interest. In common with other studies of phenotypic plasticity (Schlichting, 1986), statistical significance of the genotype term (G) was interpreted as evidence of genetic variation in the trait of interest (in the present case herkogamy), significance of the treatment effect (E) as indication of environmental effects, and its interaction (G × E) as evidence of genetic variation for plasticity.

RESULTS

Trait correlations and comparisons among the floral morphs

In the sample of 18 trimorphic Brazilian populations, the correlation structure of floral and vegetative characters indicated that several traits were significantly associated at both the phenotypic and genetic (maternal family) levels (Table 1). For example, large flowers were associated with large plant size, more flowers per inflorescence and large nectar guides at the phenotypic level, and, with the exception of plant size, these correlations were also evident at the family level. Nectar-guide size varied positively with flower number at both the phenotypic and genetic level, and with petal size at the phenotypic level only. Greater stigma–anther separation was correlated with larger perianth size at both phenotypic and genetic levels, but this trait was only associated at the phenotypic level with larger plants, more flowers per inflorescence and larger nectar guides.

The only consistent difference in floral traits among morphs within trimorphic and dimorphic populations was stigma–anther separation. In trimorphic populations, flowers of the L-morph had the largest mean herkogamy value; 4.90 mm ± 0.06 (mean ± s.e.) compared with 3.24 ± 0.07 and 2.81 ± 0.07 for the M- and S-morph (P < 0.0001 for all morph comparisons, n = 220, 293 and 105 individuals for the L-, M- and S-morph, respectively). In dimorphic populations, herkogamy in the L-morph had a similar value (4.82 ± 0.07, n = 174) to the L-morph in tristylos population. However, the M-morph had a significantly lower stigma–anther separation (1.85 ± 0.07, n = 307), which was significantly different from the L-morph (P < 0.001).

Perianth size (mm²) was not significantly different among morphs in either trimorphic (L-morph, 727.28 ± 14.46; M-morph, 699.03 ± 10.93; S-morph, 744.88 ± 16.17) or dimorphic (L-morph, 650.70 ± 12.45; M-morph, 660.30 ± 7.87; P = 0.33) populations. In trimorphic populations nectar guides were significantly larger in the M-morph than the S-morph (P = 0.041), but not different from the L-morph (L-morph, 7.42 ± 0.16; M-morph, 6.93 ± 0.11; S-morph, 7.57 ± 0.22). Both L- and M-morphs had similar-sized nectar guides in dimorphic populations (L-morph, 5.72 ± 0.17; M-morph, 5.55 ± 0.13; P = 0.5).

Patterns of herkogamy in the M-morph

An unusual feature of variation in herkogamy in E. paniculata is the bimodal distribution of stigma–anther separation in the M-morph. The top part of Fig. 2 illustrates the frequency distribution of this measurement among the three morphs. Bimodality is evident in the M-morph but not the remaining morphs. No morph exhibits bimodality for perianth size (Fig. 2, bottom part). Further examination of the bimodal nature of herkogamy in the M-morph, using the mixed-distribution (n = 2365 flowers), revealed that the observed and predicted distributions did not differ significantly from each other (χ² = 16.275, d.f. = 10, P = 0.92). This indicates that a combination of two normal curves provides a reasonable approximation to bimodality. Based on the mixed-distribution analysis, herkogamy in the M-morph can be conveniently divided into two classes: flowers with a mean herkogamy of
3.57 mm (s.d. = 0.90), and those with a mean herkogamy of 0.68 mm (s.d. = 0.63). The relative frequency of these two classes in the entire sample was 58.3% and 41.7%, respectively. As in previous studies (e.g. Barrett and Harder 1992), flowers with a herkogamy closer to the lower mean (i.e. near zero stigma–anther separation) are referred to as modified, and those with greater distances (stigma–anther separation near 3.57) as unmodified.

Relationship between herkogamy of the L- and M-morph in segregating families

The present analysis of segregating families indicated a small but significant correlation between herkogamy in flowers of the L- and M-morph (ρ = 0.239, P = 0.011, n = 111 families). To distinguish the association between herkogamy and flower size, a partial Spearman-rank correlation analysis was conducted (Kim and Yi, 2007). This analysis indicated that the coefficients of herkogamy in the L- and M-morphs are not significantly correlated after accounting for correlations with perianth size (ρ = 0.176, P = 0.062, n = 111). Moreover, Figs 2 and 3 illustrate that flowers of the M-morph commonly exhibit herkogamy values near zero, a pattern not observed in the L-morph. Indeed, families that produce M-morph flowers with small stigma–anther separation (i.e. <2 mm, ‘modified’ flowers) have the same herkogamy in the L-morph as families that produce M-morph flowers with large stigma–anther separation (i.e. >2 mm, ‘unmodified’ flowers) (W = 1459, n = 111, P = 0.575; Fig. 3). These results indicate that the modification in stigma–anther separation in E. paniculata is largely morph specific.

Patterns of variation in herkogamy among populations, families and individuals in the M-morph

The occurrence of modified flowers of the M-morph, here treated as a binary representation of herkogamy variation, was significantly different among the three population morph structures (χ² = 29.29, d.f. = 2, P < 0.0001; Fig. 4). Back transformation of the logistic regression parameters allowed the expected probability of modified flowers in each population morph structure to be calculated (Table 2A). The predicted probability of producing modified flowers in trimorphic, dimorphic and monomorphic was 26%, 67.3% and 99.3%, respectively. Mean observed values were 16.60%, 59.32% and 89.80%, respectively. The difference between observed and predicted values is likely to be due to the significant contribution of population identity, family and individuals within families to variation in herkogamy (Table 2B). Segregation of different genes within families and micro-environmental conditions in the glasshouse probably contribute to the expression of herkogamy. In contrast, flower size contributed only marginally to the probability of herkogamy modification (χ² = 3.46, d.f. = 1, P = 0.062) and its contribution was relatively small (Table 2A).

Among the 685 plants of the M-morph included in the total sample, 404 (58.97%) produced at least one modified flower. Among these plants, 231 produced only modified flowers with the remainder (173) exhibiting developmental instability producing both modified and unmodified flowers. However, these specific values should be interpreted with caution due to the small number of flowers measured per individual.

![Fig. 3](image1.png)

![Fig. 4](image2.png)
However, developmental instability was also evident among genotypes used in the phenotypic plasticity experiment described below. Because of the much larger number of flowers measured on each genotype it was possible to construct frequency histograms to illustrate the contrasting patterns of herkogamy variation (Fig. 5). Three genotypes are contrasted, each of which illustrates a distinct pattern of herkogamy variation. Genotypes (B191-3-5) and (B182-9-1) were developmentally stable, producing flowers with large versus small herkogamy values, respectively. In contrast, genotype (B189-7-1) exhibited marked developmental instability producing a bimodal distribution of herkogamy values.

Phenotypic plasticity in the expression of herkogamy

Plants of the M-morph grown in smaller pots with lower fertilizer dosage were smaller ($t = 16.72$, d.f. = 63.89, $P < 0.0001$) and produced more modified flowers than those that were grown with higher resource supply (LRT of treatment effect; $\chi^2 = 8.45$, d.f. = 1, $P = 0.004$; proportion of modified flowers $= 0.721 \pm 0.002$ and $0.561 \pm 0.003$, for high- and low-stress environments, respectively) demonstrating plasticity in the expression of herkogamy. Genotypes differed significantly in their relative production of modified flowers, as expected based on the population survey ($\chi^2 = 1164.32$, d.f. = 9, $P < 0.001$). Genotypes from a trimorphic population produced mostly unmodified flowers, whereas genotypes from a monomorphic population produced largely modified flowers. Genotypes from dimorphic populations displayed a wide range of floral modification ($0.260-0.995$ and $0.726-1$ in the low- and high-stress environments, respectively; Fig. 6). The significant genotype $\times$ environment interaction (treatment $\times$ genotype effect; $\chi^2 = 20.62$, d.f. = 9, $P = 0.014$) indicates

### TABLE 2. Analysis of the occurrence of modified flowers in the M-morph (stigma–anther separation < 2 mm) in Brazilian populations of Eichhornia paniculata

<table>
<thead>
<tr>
<th>Population type</th>
<th>Proportion of modified flowers</th>
<th>Regression estimate</th>
<th>s.e.</th>
<th>z-value</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Trimorphic</td>
<td>0.026</td>
<td>-3.590</td>
<td>0.678</td>
<td>-5.294</td>
<td>&lt;0.0001</td>
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<td>Dimorphic</td>
<td>0.073</td>
<td>0.725</td>
<td>0.809</td>
<td>5.333</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Monomorphic</td>
<td>0.093</td>
<td>4.959</td>
<td>1.475</td>
<td>3.794</td>
<td>&lt;0.0001</td>
</tr>
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<td>Perianth size</td>
<td></td>
<td>0.001</td>
<td>0.0006</td>
<td>-1.924</td>
<td>0.054</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Effects</th>
<th>Log likelihood</th>
<th>$\chi^2$</th>
<th>d.f.</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Full model</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Population</td>
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<td>30.50</td>
<td>1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Family</td>
<td>-971.67</td>
<td>37.84</td>
<td>1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Individual</td>
<td>-1035.73</td>
<td>165.96</td>
<td>1</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Sample sizes: populations $n = 29$, families $n = 186$, plants $n = 685$, flowers $n = 2365$. Models were fitted using logistic mixed-effects regression.
genetic variation for phenotypic plasticity in stigma–anther separation among the ten genotypes. Consistent with the results of the population survey, perianth size was not a significant determinant of the expression of modified flowers ($\chi^2 = 0.44$, d.f. = 1, $P = 0.507$).

**DISCUSSION**

The most significant alteration to floral architecture during the early stages of the evolution of selfing in Brazilian populations of *Eichhornia paniculata* is a reduction in herkogamy in the M-morph (Fig. 2). The results indicate that changes in herkogamy are largely independent of modifications to other floral traits, including flower size. An analysis of stigma–anther separation in segregating families of the M-morph confirmed that modifications to herkogamy are morph-specific. It was also found that the production of modified flowers was influenced by environmental conditions in some genotypes, with higher proportions produced under more stressful conditions. The ability to alter herkogamy independently of other floral characters and the plasticity of this trait has implications for the regulation of mating patterns in a species adapted to ephemeral environments (Barrett and Husband, 1997). Below are discussed the mechanisms influencing the expression of selfing modifications in *E. paniculata* and the ecological context in which changes to mating patterns occur.

**Flowers in animal-pollinated plants**

Flowers in animal-pollinated plants are made up of correlated traits that function in an integrated manner to promote pollination and mating (Berg, 1960; Armbruster *et al.*, 2004; Harder and Barrett, 2006). An important issue associated with the transition from outcrossing to selfing is to what extent individual traits can be modified independently of other traits during floral evolution (Fishman *et al.*, 2002). The results indicate that in all style morphs of *E. paniculata*, perianth size, nectar guide size, flower number and herkogamy are positively correlated among each other at the phenotypic level, although at the family level only the correlations between perianth size, herkogamy and nectar guide size are significant (Table 1). Phenotypic and genetic correlations
among floral traits are generally expected to occur because of
shared developmental pathways (Krizek and Fletcher, 2005)
and strong stabilizing selection for floral integration
(Armbruster et al., 2004). Functional integration would be
especially likely in a heterostylos species, such as
*E. paniculata*, where reciprocity of sex-organ position
promotes disassortative pollination among the style morphs by
long-tongued bees.

The positive correlation between stigma–anther separation
and perianth size in trimorphic populations of *E. paniculata*
might suggest that genetic modifications to stigma–anther sep-
oration would depend, in part, on changes to flower size.
Indeed, this association commonly occurs in other groups
where reductions in herkogamy are often associated with
the evolution of smaller flowers (Fishman et al., 2002).
However, the present results indicate that during the early
stages of the establishment of selfing this association does
not occur and that stigma–anther separation can be altered
independently of changes to flower size (Table 2). Earlier,
Fenster and Barrett (1994) reported weak correlations
(Pearson’s correlation coefficients <0:25) between perianth
width and the length of stamen filaments. Since herkogamy
modification is mostly due to elongation of short-level
stamen filaments (Seburn et al., 1990; Richards and Barrett,
1992) weak associations between perianth characteristics and
herkogamy are not unexpected. Accordingly, the present
results show that variation in herkogamy among the floral
morphs is not paralleled by changes in perianth size (Fig. 2),
and the analysis of the production of modified flowers in the
M-morph demonstrate that whether a flower is modified or
not is not influenced by perianth size (Table 2). Collectively
these results support the conclusion that early stages in the
evolution of the selfing syndrome in *E. paniculata* are initiated
by relatively simple changes to stamen position that
occur independently of modifications to other floral traits.
However, once selfing is firmly established, selection for
reduced flower size may often proceed, as seems likely to
have occurred in the highly autogamous populations of
*E. paniculata* in Jamaica and Cuba, which possess
much smaller flowers than occur in north-east Brazil. These
changes to flower size in selfing populations are likely to be
a response to selection for a reduction in the organs of attrac-
tion and a more economical use of floral resources.

**Morph-specific expression of reduced herkogamy**

The reduction in herkogamy associated with the transition to
self-fertilization in Brazilian populations of *E. paniculata* is
largely restricted to individuals of the M-morph (Fig. 2). Stigma–anther separation in long-styled individuals was not
influenced by whether their mid-styled siblings have modified
or unmodified flowers. This lack of correspondence demon-
strates morph-limited expression of selfing adaptations in
*E. paniculata*. Morph-specific expression in this species may
arise because different anther levels are involved in determin-
ing any potential reduction in herkogamy in the M- and
L-morphs (see Fig. 1). It is likely that in this heterostylous
species mutations causing filament elongation are specific to
particular stamen levels and are simply not expressed in alter-
nate morphs. In the present study, the position of short-level
anthers in flowers of the L-morph was not measured. Instead
attention was paid to the distance separating the closest mid-
level anther and stigma of long styles. However, a previous
investigation failed to find evidence of elongation of short-
level anthers in flowers of the L-morph among families con-
taining selfing variants of the M-morph (Fenster and Barrett,
1994). This supports the interpretation that the mutations mod-
ifying short-level stamens in the M-morph are not expressed in
either of the two stamen levels of the L-morph. Field obser-
vations are also consistent with morph-specific expression of
herkogamy variation. In dimorphic populations in north-east
Brazil, Jamaica and Cuba individuals of the M-morph com-
monly (Brazil) or exclusively (Caribbean), produce modified
flowers whereas those of the L-morph maintain significant
stigma–anther separation.

Small-flowered disjunct populations of *E. paniculata* in
Mexico and Nicaragua are composed exclusively of semi-
homostylos long-styled plants. This demonstrates that in
other parts of the geographical range the evolution of selfing
is not restricted to the M-morph (Barrett, 1996; S. C. H.
Barrett, unpubl. res.). Preliminary genetic studies of these
semi-homostylos variants of the L-morph suggest that floral
modifications promoting selfing result from quantitative
inheritance, unlike the self-pollinating forms of the M-morph
from Brazil in which stamen elongation is simply inherited
(Fenster and Barrett, 1994; M. Vallejo-Marín and S. C. H.
Barrett, unpubl. res.). Why selfing modifications are restricted
to the M-morph in Brazilian and Caribbean populations is
unclear. It has been suggested that the likelihood of selfing
evolving in the L-morph may be contingent on whether the
M-morph is present in populations because of the speed in
which selfing can evolve under polygenic versus major gene
control (Barrett, 1996). Ongoing work is investigating the
genetic architecture and evolutionary dynamics of selfing in the
L- and M-morphs. In particular, it will be important to
determine the role of genetic and developmental factors in
causing morph-limited expression of short-stamen modifi-
cation in the M-morph.

**Variation in the incidence of self-pollinating flowers**

The incidence of reduced herkogamy in the M-morph of
*E. paniculata* varies among populations with different morph
structures. Modified flowers are less common in tristylos
populations, abundant in dimorphic populations, and largely
fixed in monomorphic populations (Fig. 4 and Table 2B). This
pattern is associated with the evolutionary breakdown
of tristyly and the transition from outcrossing to selfing.
However, appreciable frequencies of self-pollinating flowers
of the M-morph (mean 16.60%; range 0–36.8%) were
recorded among the 18 tristylos populations that were exami-
ned under uniform glasshouse conditions. Elsewhere it has
been proposed that the joint action of stochastic forces and
natural selection can explain the loss of morph diversity and
spread and fixation of self-pollinating variants in dimorphic
and monomorphic populations of *E. paniculata*, respectively
(Barrett et al., 1989; Husband and Barrett, 1992). The glass-
house comparisons demonstrate that selfing modifiers are rep-
resented among the standing genetic variation of largely
outcrossing tristylos populations. The facility for self-
pollination would enable modified plants of the M-morph to found colonies following dispersal events from trimorphic populations. Founders that are heterozygous at the M-locus would produce L- and M-styled plants and this may account for the origin of some dimorphic populations.

The glasshouse comparisons were not specifically designed to measure the heritability of modified flower production in M-styled plants. In addition, the experiments did not explicitly account for potential maternal effects. However, variation in the incidence of modified flower production among families grown under uniform conditions (Table 2B) provides evidence that this trait has a genetic basis. Together with the significant variation among genotypes detected in the plasticity experiment (Fig. 6), and the analysis of segregation patterns in controlled crosses between modified and unmodified plants (Fenster and Barrett, 1994, M. Vallejo-Marín and S.C.H. Barrett, unpubl. res.), there seems little doubt that herkogamy variation in *E. paniculata* has a genetic component. The variation detected within segregating families of the M-morph indicates that flowers produced by siblings are often more different than flowers produced within individuals (Table 2B). This variation among siblings could be due to subtle microenvironmental variation in the glasshouse and/or segregation of different alleles within families. Controlled crosses suggest that herkogamy modification is due to a small number of recessive factors (Fenster and Barrett, 1994). The segregation of stamen-modification alleles in heterozygous families may be responsible for some of the variation observed among siblings.

**Phenotypic plasticity and developmental instability of floral traits**

Floral traits commonly display relatively uniform morphological expression resulting from developmental homeostasis (reviewed in Fenster and Galloway, 1997) and stabilizing selection (Armbruster et al., 2004). A consistent correspondence between sexual organs and pollinator positioning is necessary to ensure effective pollen transfer, especially in heterostylous species (Lloyd and Webb, 1992). In this context, plasticity and/or developmental instability of floral characters is most often considered maladaptive and few studies have considered the potential benefits of these forms of intra-plant variation in reproductive traits (but see Lloyd, 1984). Although the present sample of genotypes was relatively small (*n* = 10), variation among genotypes was detected in the plasticity of herkogamy (Fig. 6). Variation ranged from strong centralization with almost identical proportions of modified flowers between the two growing conditions (e.g. B191-3-5, B191-5-1, B182-5-6, B182-9-1, B177-7-5 and B180-8-4), to an increase in the proportion of modified flowers in B186-9-7 and B189-7-1 from 26% to 76% and 57% to 78%, respectively, from low- to high-stress environments.

Several plants investigated produced bimodal distributions of stigma–anther separation values (Fig. 5; see Seburn et al., 1990; Barrett and Harder, 1992). This pattern results from the production of two classes of flowers by a single individual: modified flowers with herkogamy values near zero and unmodified flowers with higher herkogamy values. The bimodality is mainly due to differences in filament elongation, which occur through changes in cell elongation 24 h prior to anthesis (Richards and Barrett, 1992). No other example is know in the literature in which a species produces this pattern of herkogamy variation. It can be viewed as a form of developmental instability, but also as an expression of phenotypic plasticity because environmental conditions modify the frequencies of modified and unmodified flowers.

Developmental instability can be influenced by a variety of internal and external factors including homozygosity, mutation, breakdown of adapted gene complexes, and environmental stress (for a recent review of developmental instability, see Polack, 2003). In *E. paniculata*, developmental instability of stamen position in the M-morph of *E. paniculata* is not easily explained by architectural (position) effects (Diggle, 2003), as the probability of producing a modified flower depends on a complex interaction between plant identity, floral position, inflorescence identity, age of the plant and environmental conditions (Barrett and Harder, 1992). Developmental instability could result from the increased selfing that is characteristic of selfing variants in dimorphic populations (Barrett et al., 1989). Inbreeding is thought to reduce the ability of genetic systems to buffer against environmental and developmental noise (Lerner, 1954; Jinks and Mather, 1955; Rendel, 1959; Levin, 1970; Fenster and Galloway, 1997). Another possibility is that developmental instability results from incomplete expression of mutations modifying stigma–anther separation (i.e. partial expressivity; Suzuki et al., 1986, p. 71; Richards and Barrett, 1992). Novel mutations may display variable expression when they first arise because their interactions with the rest of the genome have not been canalized by selection (see Waddington, 1942; Schmalhausen, 1949; Fenster and Galloway, 1997; De Visser et al., 2003). The fate of incomplete expressivity is often determined by selection acting on additional modifier genes that regulate trait expression resulting in either full or partial expressivity (Levin, 1970). This hypothesis predicts that developmental instability should be highest in populations where herkogamy mutations have recently spread (e.g. dimorphic populations) compared with those in which herkogamy mutations have been refined by selection (monomorphic populations). Field observations and glasshouse comparisons of genotypes from dimorphic and monomorphic populations generally support this hypothesis (S.C.H. Barrett, unpubl. res.).

Variation in herkogamy resulting from either phenotypic plasticity and/or developmental instability could allow individuals to modify rates of self- and cross-fertilization to match current environmental conditions. An increase in self-fertilization may be favoured in more stressful environments where either biotic (e.g. pollinator abundance) or abiotic conditions (e.g. water availability causing reduced density) limit cross-pollination (Stebbins, 1957; Elle and Hare, 2002; Elle, 2004; Moeller and Geber, 2005). Populations of *E. paniculata* in north-east Brazil grow in ephemeral pools and inundated areas where growing conditions change rapidly (Barrett and Husband, 1997). Increased selfing may be favoured in stressful environments experiencing drought. Under these conditions small plant stature and small population sizes could make it difficult to attract pollinators to ensure seed set. Plastic increases in autonomous self-
pollination could then confer reproductive assurance in such populations. In contrast, plants growing in more favourable (e.g. wetter) environments are generally larger in size with more showy floral displays and may be able to attract sufficient pollinators to outcross successfully. To the extent that heterogeneous environments favour different mating strategies, within-plant variation of herkogamy in *E. paniculata* may represent an adaptive strategy in which individuals produce more than one functional class of reproductive organ, i.e. a multiple strategy *sensu* Lloyd (1984). Similar variation is evident in the production of self-fertilizing (cleistogamous) and open-pollinated (chasmogamous) flowers in many taxa (reviewed in Oakley *et al.*, 2007). Elsewhere, discrete morphological variation in reproductive organs within individuals, e.g. somatic polymorphisms such as andromonoecy (male and hermaphrodite flowers), gynomonoecy (female and hermaphrodite flowers) and heterothaly (two kinds of anthers in the same flower) – has evolved repeatedly in the evolutionary history of angiosperms. Future field studies are required to investigate whether within-plant variation of herkogamy in *E. paniculata* has any adaptive value.

**SUPPLEMENTARY DATA**

Supplementary information is available online at www.aob.oxfordjournals.org and includes the following tables. Table S1: Populations of *Eichhornia paniculata* (Pontederiaceae) from north-east Brazil that were used in this study. Table S2: Population of origin, number of ramets, and total number of flowers of Brazilian genotypes of *Eichhornia paniculata* measured from the two environmental treatments in the phenotypic plasticity experiment.

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**LITERATURE CITED**


