

Delayed autonomous selfing and inbreeding depression in the Australian annual *Hibiscus trionum* var. *vesicarius* (Malvaceae)

Leahwyn Seed^A, Glenda Vaughton^{A,B} and Mike Ramsey^A

^ABotany, University of New England, Armidale, NSW 2351, Australia.

^BCorresponding author. Email: gvaughto@une.edu.au

Abstract. The Australian annual, *Hibiscus trionum* var. *vesicarius*, produces large, showy flowers typical of an outcrossing species, yet flowers autonomously self-pollinate. We used experimental pollinations to examine self-compatibility, inbreeding depression and the efficiency and mechanism of autonomous selfing. Seed set of self- or cross-pollinated flowers did not differ, indicating that plants were fully self-compatible. Seed set following autonomous selfing varied among plants, and was 11–103% of that following hand-selfing. Autonomous selfing was delayed, and styles curved and stigmas contacted the anthers before flowers closed. Delayed selfing was facultative and curvature depended on the number of pollen grains on stigmas, with 50 or more grains preventing curvature. Both self- and cross-pollen prevented curvature. Similarly to unpollinated styles, styles that were pollinated with dead pollen curved fully, indicating that either pollen germination or pollen-tube growth prevents curvature. Within flowers, the five styles acted independently, depending on the amount of pollination that each received. Although plants exhibit a high potential for selfing, crossed progeny outperformed selfed progeny and cumulative inbreeding depression was 0.64, which is high for a self-compatible annual. Despite this high inbreeding depression, delayed selfing would be advantageous under variable pollinator conditions, providing reproductive assurance.

Introduction

Despite theoretical predictions that plant mating systems should be predominantly selfing or outcrossing, mixed mating systems are common in plant populations (Lloyd and Schoen 1992; Holsinger 1996; Vogler and Kalisz 2001). In self-compatible plants, mixed mating often occurs as a result of selfing by pollinator-mediated geitonogamy and autogamy, which are considered to be unavoidable accompaniments to outcrossing (Lloyd and Schoen 1992). Another mechanism that can result in mixed mating is outcrossing combined with autonomous self-pollination that occurs without the aid of external agents (Lloyd and Schoen 1992; Herlihy and Eckert 2004).

Three modes of autonomous self-pollination were recognised by Lloyd (1979). Prior selfing occurs before opportunities for outcrossing, competing selfing occurs at the same time as outcrossing and delayed selfing occurs after opportunities for outcrossing. Both prior and competing selfing can lower the potential for outcrossing. By contrast, delayed selfing allows outcrossing when pollinators are present, but provides reproductive assurance when pollinators are absent or in low abundance (Lloyd 1979, 1992; Lloyd and Schoen 1992). Of the three modes, delayed selfing is considered especially advantageous when pollinator visitation, and hence outcross pollen receipt, is

unpredictable within and among seasons. Confronted with such unpredictability, plants with delayed selfing experience the ‘best of both worlds’ and can produce a mixture of selfed and outcrossed seeds, depending on the amount of cross pollen delivered by pollinators (Becerra and Lloyd 1992; Kalisz and Vogler 2003; Kalisz *et al.* 2004).

Reproductive assurance is expected to be of particular importance for annual plants because they have only one opportunity to reproduce. If unfavourable conditions during flowering cause pollination failure, then the reproductive loss cannot be made up in subsequent seasons (Schoen and Brown 1991; Lloyd 1992; Lloyd and Schoen 1992; Kalisz and Vogler 2003). Delayed selfing provides reproductive assurance, and during periods of low pollinator visitation shifts occur in mating from outcrossing to autonomous selfing (Kalisz *et al.* 2004). The realised benefit of reproductive assurance, however, will depend not only on the efficiency of autonomous selfing, but also on the magnitude of inbreeding depression (Husband and Schemske 1996). Although high inbreeding depression can reduce the benefit, providing that some selfed seeds survive to reproduce, delayed selfing will act to increase plant fitness either by ensuring seeds are produced or by augmenting seed production resulting from previous cross-pollination (Lloyd 1992; Lloyd and Schoen 1992).

Mechanisms of delayed selfing frequently involve movements of floral parts that bring the anthers and stigmas into physical contact at the end of floral life. Flowers are often herkogamous during the period when outcrossing occurs, but floral movements during senescence ensure self-pollination. Such movements may involve movement of the anthers towards the stigma (*Aquilegia canadensis*, Eckert and Schaefer 1998; *Kalmia latifolia*, Rathcke and Real 1993; *Crotalaria micans*, Etcheverry *et al.* 2003), or the stamens may be dragged past the stigma as the corolla abscises (*Mimulus guttatus*; Dole 1990). Alternatively, styles may curl downward so that the stigmas contact the anthers or the pollen presentation structures as in certain members of the Asteraceae, Campanulaceae, Malvaceae and Scrophulariaceae (Faegri and van der Pijl 1979; Buttrose *et al.* 1977; Kalisz *et al.* 1999). Although delayed selfing often occurs regardless of prior cross-pollination, floral movements can also be facultative depending on whether stigmas are pollinated. For example, in *Hibiscus laevis* style branches remain upright if pollinated, but curve downwards contacting the anthers if they are not pollinated (Klips and Snow 1997).

Here we extend previous work by Buttrose *et al.* (1977) and Ramsey *et al.* (2003) on the breeding system of *Hibiscus trionum* (Malvaceae). In Australia, *H. trionum* var. *trionum* is an introduced annual weed and *H. trionum* var. *vesicarius* is a native annual. Buttrose *et al.* (1977) examined style curvature in replicates of one genotype, probably of *H. trionum* var. *trionum*, although the variety was not reported (see figs 1–3 in Buttrose *et al.*). Also, in *H. trionum* var. *trionum*, Ramsey *et al.* (2003) found high levels of autonomous selfing and low levels of inbreeding depression, as expected for an invasive agricultural weed. In the current study, we focus on *H. trionum* var. *vesicarius*, the native taxa, and investigate the mechanisms and possible benefits of delayed autonomous selfing. To accomplish this, we assess the level of self-compatibility, examine the fitness of selfed progeny relative to crossed progeny at a range of life-cycle stages, and estimate inbreeding depression. We then assess variation in the effectiveness of autonomous selfing among several different genotypes. Finally, we examine the mechanism of autonomous selfing and consider whether (1) prior pollination prevents style curving, (2) styles act independently of each other and (3) the degree of style curvature is related to the amount of pollen deposited onto stigmas.

Materials and methods

Study species

Two varieties of *Hibiscus trionum* (Malvaceae) are currently recognised in Australia; var. *trionum*, the narrow-leaved form, is an introduced weed of cultivated and disturbed sites; and var. *vesicarius*, the wide-leaved form, is native to northern and inland areas of Australia (Harden 2000). There are pronounced phenetic and life-history differences between

these varieties that remain unchanged in F₁ and F₂ generations and when grown under common garden conditions (Johnson *et al.* 2003; G. Vaughton and M. Ramsey, unpubl. data). Moreover, inter-variety crosses do not yield seeds, indicating that these taxa may be different species rather than varieties (Seed 2003). Although taxonomic revision is required, we follow current treatments and refer to the two taxa as varieties (Harden 2000).

Hibiscus trionum var. *vesicarius* Hochr. is often found on clay soils after flooding or heavy rains. At our study site, flowers are white in colour, with a pale-yellow centre (Harden 2000; Johnson *et al.* 2003). Flowers open in the morning, close by mid-afternoon and are large and attractive (mean diameter \pm s.e., 36.6 \pm 0.8 mm, n = 30 flowers). The numerous stamens are fused into a column surrounding the style. The pistil has five styler branches, each with a capitate stigma. Introduced honeybees, native bees, flies and butterflies visit flowers. Insects move among plants, potentially cross-pollinating flowers. The fruit is a 5-valved capsule with up to 50 seeds (Seed 2003).

Plants for this study were established from seeds collected 17 km west of Narrabri, NSW (30° 12' S, 149° 39' E, 214 m a.s.l.) in December 2002. One fruit was collected from each of 48 plants that were growing at least 5 m apart. Seeds were stored in individual paper envelopes for at least 1 month before being used. To establish experimental populations, we used one seed from each of 15 of the 48 field-collected seed families. With a scalpel, we hand-scarified seeds at the hilum to break the seed-coat-imposed dormancy. Seeds were then placed in Petri dishes on germination pads moistened with distilled water (Ramsey *et al.* 2003). Shortly after seeds germinated, seedlings were planted in pots containing sand, loam and peat (1 : 1 : 1). Pots were placed on glasshouse benches. Plants were watered regularly and fertilised every 3 weeks with liquid Aquasol[®] and once with slow-release Osmocote[®], unless otherwise stated.

Self-compatibility

To assess self-compatibility, we assigned eight newly opened flowers on each of 15 plants to either hand self- or hand cross-pollination treatments. On each plant, treatments were randomised in time. Selfed flowers received pollen from the same flower. Crossed flowers were emasculated and then pollinated with pollen from another plant. We used newly opened flowers and ensured that stigmas were thoroughly pollinated. After 5 weeks the fruits were dissected and the numbers of mature seeds were counted. Numbers of selfed or crossed seeds per fruit were compared with a two-way ANOVA, with pollination treatment and maternal plants as fixed and random factors, respectively. On 10 different plants, we tested for agamospermy by emasculating two flowers on each plant. No seeds were produced, indicating that agamospermy does not occur. During all experiments, the glasshouse was free of pollinating insects.

Inbreeding depression

To examine inbreeding depression we randomly selected 8 of the 15 maternal plants used to assess self-compatibility and used the selfed and crossed seeds from each family. Because replicate cross-pollinated fruits potentially had different fathers, we mixed the seeds of the four crossed fruits from each plant thoroughly. We also mixed the seeds of the selfed fruits from each plant. We individually weighed 10 selfed and 10 crossed seeds from each family to the nearest 0.1 mg. We then hand-scarified 18 selfed and 18 crossed seeds from each family, and placed seeds on moist germination pads in Petri dishes. Dishes were arranged randomly in a germination cabinet set at 20°C and a 12 h light : 12 h dark photoperiod. We recorded the number of germinated seeds over the next 10 days, by which time all seeds had germinated. Selfed and crossed seeds germinated equally over this period, but because we hand-scarified seeds, the speed of germination may not reflect that which occurs under natural conditions.

For each maternal family, four selfed and four crossed seedlings were randomly chosen and planted into 1140-cm³ pots with a 1 : 1 : 1 mixture of sand, loam and peat. Pots were arranged on glasshouse benches as four blocks, with selfed and crossed seedlings from each maternal plant represented in each block. Plants were watered every 2–3 days and fertilised with 50 mL of Aquasol once after 2 weeks. After 14 weeks, numbers of flowers and fruits per plant were counted. One fruit was harvested from each plant and numbers of seeds were counted. Vegetative biomass (stems, leaves) and reproductive biomass (fruits, seeds, flowers) were determined after drying at 65°C for 14 days. Vegetative biomass was considered an indicator of future reproductive potential.

All self- and cross-fertilised progeny germinated and survived, and we did not analyse these data. For the other traits, we used ANOVAs to examine differences between the two pollination treatments. Pollination was a fixed factor, and maternal family was a random factor. Block was a random factor for analyses of traits derived in the glasshouse. If necessary, data were transformed to ensure homogeneity of variances and normality. In these analyses, pollination × maternal family interactions test whether the differences between selfed and crossed values vary among families, but they do not test whether families differ in the level of inbreeding depression. To accomplish this, we followed Johnston and Schoen (1994) and transformed the original data to a logarithmic scale to assess the significance of the pollination × family interactions.

For all traits, we used individual families to calculate relative performance to be

$$RP = w_s/w_c,$$

where w_s and w_c are the mean performances of selfed and crossed progeny, respectively. We estimated inbreeding depression by using a subset of traits that are likely to be the most important to overall fitness. These included the number of seeds per fruit for maternal plants and for F₁ progeny, percentage seed germination, percentage survival to flowering, fruit number per plant, seed number per fruit and vegetative mass. Cumulative relative performance was calculated for each family as the product of the relative performances of the above six traits. Inbreeding depression was calculated to be

$$\delta = 1 - W_s/W_c,$$

where W_s/W_c is the cumulative relative performance (Charlesworth and Charlesworth 1987).

Effectiveness of autonomous v. hand self-pollination

From the 48 field-collected seed families, we grew an additional 10 plants and randomly assigned 10 and 6 flowers on each to autonomous-selfing and hand-selfing treatments, respectively. Autonomously selfed flowers were left untouched, and hand-selfed flowers were self-pollinated, as described above. After 5 weeks, fruits were dissected, and the numbers of mature seeds were counted. We used a two-way ANOVA to compare the number of seeds per fruit between pollination treatments. Pollination treatment and maternal plant were considered fixed and random factors, respectively.

Prior pollination and autonomous selfing

To examine the effect of prior pollination on autonomous selfing, we randomly assigned two flowers on each of 10 plants to each of three pollination treatments: hand self-, hand cross- or autonomous self-pollination. Newly opened flowers were hand-pollinated as described above, when styles were upright. Flowers were collected when they had closed, ~8 h after pollinations. Flowers were dissected, and the curvature of styles was examined under a dissecting microscope. We recognised the following four categories of style curvature: (1) upright, (2) slightly curved ≤45°, (3) curved between 90° and 135°, and

(4) fully curved between 135° and 180°. Only stigmas in Categories 3 and 4 touched the anthers, either partially or fully. We did not observe styles between Categories 2 and 3.

On another 10 plants, we assessed the effects of dead pollen and emasculation on style curvature. On each plant, we randomly assigned two flowers to each of four pollination treatments. Stigmas were (1) pollinated with dead pollen, (2) pollinated with fresh self pollen, (3) unpollinated and anthers were removed, and (4) unpollinated and anthers were left intact. We killed pollen by heating it in a microwave oven. Style curvature in each treatment was recorded.

In both experiments, styles of the two flowers in each treatment behaved similarly and were pooled to increase sample sizes for analyses. We used logistic models with a binomial error structure and a logit-link function to examine the effects of treatment and plant as explanatory variables on the number of styles in each curvature category, the response variable. We scaled deviances to remove overdispersion and used deviance ratios, a measure that approximates *F*-ratios (Crawley 1993). Significant treatment × curvature or plant × curvature interactions would indicate that the number of styles in the categories varied depending on pollination treatment or plant, respectively. Analyses were computed with GLMStat (Beath 2000).

Style independence within flowers

To examine whether the five styles within flowers curve independently of each other we pollinated either zero, one, two, three, four or five stigmas, leaving the remaining stigmas unpollinated. Five flowers per treatment were emasculated and the stigmas of each were either self-pollinated or left unpollinated. After 6 h, flowers were dissected and the curvature of styles assessed. The styles of the five flowers were pooled to ensure adequate sample sizes for analyses. When either no stigmas or all stigmas were pollinated, all of the styles either curved or remained upright, respectively. Consequently, we omitted these treatments from the statistical analyses because including them would have increased the probability of obtaining significant results. We undertook two separate analyses. First, using all data, we computed a test of independence (4 × 4 contingency table) to examine whether the frequency of styles in the four curvature categories (described above) varied depending on the number of styles pollinated within flowers (1, 2, 3 or 4 stigmas pollinated per flower). If styles act independently, then we predict that the frequency of styles that were curved or upright in each category depends on pollination treatment, as indicated by a significant treatment × category interaction. Second, we computed separate goodness-of-fit tests for each pollination treatment to examine whether the observed frequencies of curved and upright styles differed from the expected frequencies. We estimated expected frequencies assuming that pollinated styles would remain straight or slightly curved and not contact the anthers (Categories 1 and 2), whereas unpollinated styles would curve to contact the anthers (Categories 3 and 4). If styles act independently, then we predict close concordance between the observed and expected frequencies, and non-significant goodness-of-fit tests. For analyses, we used GLMStat (Beath 2000).

We also assessed whether seed set depended on the number of styles that were pollinated. To achieve this, we pollinated either one or five stigmas per flower. Four flowers were randomly assigned to each treatment on each of 10 plants. Flowers were emasculated and then stigmas were self-pollinated. The mean (±s.e.) number of pollen grains deposited per stigma (103.0 ± 4.5, *n* = 20) greatly exceeded the number of ovules per flower (mean ± s.e.; 48.5 ± 0.5, *n* = 135). Seed set was assessed, and treatments were compared with a two-way ANOVA.

Relationship between pollen deposition and style curvature

We pollinated individual stigmas with varying amounts of pollen and assessed style curvature. Forty flowers were emasculated and one stigma

on each was self-pollinated. Closed flowers were collected 6 h later, and we counted the number of pollen grains adhering to stigmas under a dissecting microscope; pollen deposition ranged from 1 to 103 grains. Style curvature was quantified by measuring the distance of the stigma from the base of the style. Large distances between the stigma and style base indicate minimum curvature of the style branches, whereas small distances indicate maximum curvature. We examined the relationship between style curvature and the number of pollen grains on stigmas by using least squares linear regression.

Results

Self-compatibility and inbreeding depression

The number of seeds per fruit did not differ following self- or cross-pollination, but seed set varied significantly among maternal families (Tables 1 and 2). Self-compatibility of the plants, quantified by dividing selfed seed set by crossed seed set, ranged from 0.81 to 1.17 (mean \pm s.e., 1.00 ± 0.02).

For the F₁ families, seed germination and plant survival of both self- and cross-fertilised progeny were 100%. For the other traits, block was not significant in any of the ANOVAs (all $F_{3,45} < 1.95$, $P > 0.13$). Maternal families differed significantly for all traits except reproductive mass (Table 1). For pollination treatments, selfed and crossed seed mass did not differ. After 14 weeks, however, crossed plants were significantly larger than selfed plants and produced more flowers, fruits and autonomous seeds per fruit (Tables 1 and 2). For all traits pollination \times family interactions were not significant (all $P > 0.05$, Table 1). Similarly, interactions were not significant when data were log-transformed (all $P > 0.05$). Relative self-performances for the fitness traits varied from 0.55 to 1.00. Inbreeding depression, estimated as the mean of the eight families, was 0.64 ± 0.02 and significantly greater than 0.50, the value above which the

Table 1. Results of ANOVAs showing the effects of pollination, maternal family and their interaction on plant traits in *Hibiscus trionum* var. *vesicarius*

Means \pm s.e. are given in Table 2. *F*-values with asterisks are significant ($^{\dagger}P < 0.10$, $^{**}P < 0.01$, $^{***}P < 0.001$)

Trait	Pollination		Source of variation		Pollination \times Family	
	d.f.	<i>F</i> -value	d.f.	<i>F</i> -value	d.f.	<i>F</i> -value
Maternal plant						
Seeds per fruit	1,14	0.04	14,90	4.90 ***	14,90	1.23
F ₁ progeny						
Seed mass	1,7	0.21	7,144	12.64***	7,144	1.92 [†]
Flowers per plant	1,7	15.27**	7,45	3.21**	7,45	1.75
Fruits per plant	1,7	16.75**	7,45	3.67**	7,45	2.00
Autonomous seeds per fruit	1,7	4.93 [†]	7,45	1.92 [†]	7,45	1.54
Reproductive mass	1,7	55.27***	7,45	0.39	7,45	1.21
Vegetative mass	1,7	134.21***	7,45	5.66***	7,45	1.18

Table 2. Performance following self- or cross-pollination for various life-cycle stages in *Hibiscus trionum* var. *vesicarius*

For seed set and the other parameters 15 and 8 maternal plants were used, respectively. Sample sizes (*n*) are the numbers of fruits, seeds or plants for each pollination treatment pooled over the maternal families. [†]denotes parameters used to estimate cumulative relative performance and inbreeding depression, based on eight plants

	<i>n</i>	Selfed	Crossed	Relative performance
Maternal plant				
Seeds per fruit	60	43.4 \pm 0.8	43.6 \pm 0.8	1.00 \pm 0.02 [†]
F ₁ progeny				
Seed mass (mg)	80	9.07 \pm 0.08	9.01 \pm 0.10	1.01 \pm 0.01
Seed germination (%)	144	100.0 \pm 0.0	100.0 \pm 0.0	1.00 \pm 0.00 [†]
Plant survival (%)	32	100.0 \pm 0.0	100.0 \pm 0.0	1.00 \pm 0.00 [†]
Flowers per plant	32	7.7 \pm 0.3	9.9 \pm 0.4	0.79 \pm 0.05
Fruits per plant	32	6.9 \pm 0.3	9.0 \pm 0.3	0.78 \pm 0.04 [†]
Autonomous seeds per fruit	32	16.6 \pm 0.9	20.5 \pm 1.6	0.84 \pm 0.07 [†]
Reproductive mass (mg)	32	1530.4 \pm 35.5	2117.9 \pm 60.3	0.73 \pm 0.03
Vegetative mass (mg)	32	721.7 \pm 31.1	1339.0 \pm 80.7	0.55 \pm 0.03 [†]
Cumulative performance				0.36 \pm 0.02
Inbreeding depression				0.64 \pm 0.02

automatic gene transmission advantage of selfing is negated (Table 2; one-tailed t -test, $t_7 = 9.14$, $P < 0.001$).

Effectiveness of autonomous v. hand self-pollination

The number of seeds per fruit following autonomous selfing was only 77% that following hand-selfing (autonomously-selfed: 27.4 ± 1.3 , hand-selfed: 35.4 ± 0.8 ; $F_{1,9} = 11.34$, $P < 0.01$). Both maternal families ($F_{9,140} = 25.45$, $P < 0.001$) and the pollination \times family interaction ($F_{9,140} = 4.84$, $P < 0.001$) were significant. Overall, autonomous-selfed seed set ranged from 11 to 103% that of hand-selfed seed set (Fig. 1).

Prior pollination and autonomous selfing

Style curvature depended on pollination treatments and whether flowers were hand-pollinated or left untouched (Fig. 2; deviance ratio, pollination \times curvature interaction: $F_{6,56} = 32.71$, $P < 0.001$). About 93% of styles curved to contact the anthers (Categories 3 and 4) in the autonomous self-pollination treatment, whereas only 19% and 8% of styles curved following hand-selfing and crossing (Category 3 only) (Fig. 2). Style curvature also depended on plant (plant \times curvature interaction $F_{21,56} = 3.09$, $P < 0.001$). All plants, however, behaved similarly in that the majority of styles either curved to touch the anthers or did not, depending on treatment.

In the second experiment, style curvature depended on pollination treatment (deviance ratio; pollination \times curvature interaction: $F_{9,108} = 341.5$, $P < 0.001$). Following pollination with fresh self-pollen, only 3% of styles curved to touch the anthers (Category 3 only), whereas 100% of styles curved when flowers were pollinated with

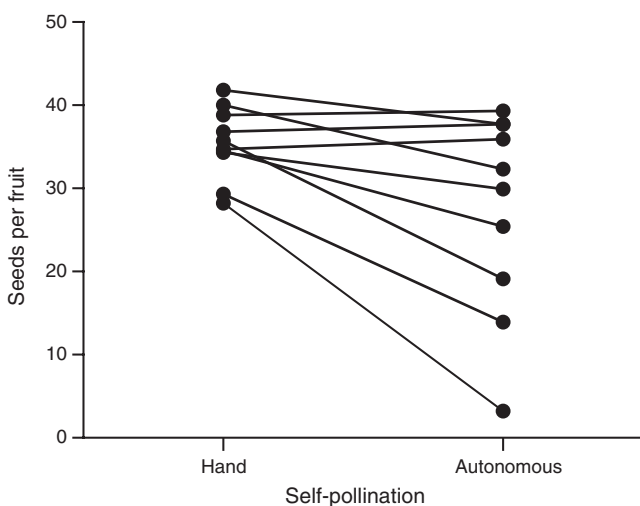


Fig. 1. Variation among plants in the mean number of seeds per fruit following hand self-pollination and autonomous self-pollination in *Hibiscus trionum* var. *vesicarius*. Six and 10 fruits were used for the hand-selfing and autonomous-selfing treatments, respectively, on each of 10 plants.

dead pollen (Categories 3 and 4). When flowers were left to autonomously self-pollinate or were emasculated and not pollinated, all (100%) styles curved (Categories 3 and 4). Curvature also depended on plant (plant \times curvature interaction $F_{27,108} = 8.11$, $P < 0.001$). Nevertheless, all plants behaved similarly in that the majority of styles remained upright following pollination with fresh pollen.

Style independence

The curving of individual styles within flowers was independent of the other styles. The frequency of styles that were curved or upright depended on pollination treatment (test of independence: $\chi^2 = 60.86$, d.f. = 9, $P < 0.001$). All 50 pollinated styles remained upright, and 45 (90%) of the 50 unpollinated styles curved fully to touch the anthers. However, when the five unpollinated styles that failed to curve were examined under a microscope, all had pollen grains deposited on the underside of their stigmas, indicating that they had been contaminated. For the second analysis, none of the goodness-of-fit tests for the four pollination treatments was significant (all $\chi^2 < 1.00$, d.f. = 1, $P > 0.350$). The observed frequency of styles that curved such that their stigmas touched the anthers did not differ from the expected frequency, indicating that styles within flowers act independently with respect to curvature.

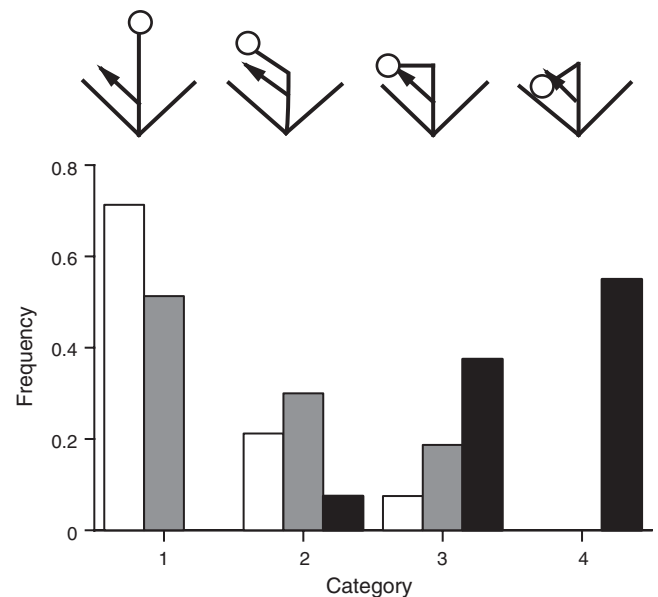


Fig. 2. Frequency of styles in the four styler curvature categories following cross-pollination (white bars), self-pollination (grey bars) and autonomous self-pollination (black bars). The four curvature categories are illustrated at the top of the frequency histogram. In this illustration, lower lines represent the corolla, and lines terminating with open circles or solid triangles represent styles and stigmas or filaments and anthers, respectively. Curvature depended strongly on whether stigmas were pollinated. Unpollinated stigmas curved to touch the anthers, resulting in delayed autonomous selfing, whereas most pollinated stigmas failed to contact the anthers.

Seed set of flowers that had one stigma pollinated was only ~50% that of flowers that had all five stigmas pollinated (mean \pm s.e., 18.8 ± 1.0 v. 36.9 ± 0.8 ; $F_{1,9} = 172.07$, $P < 0.001$). Plants differed significantly ($F_{9,60} = 6.20$, $P < 0.001$), and the pollination \times plant interaction neared significance ($F_{9,60} = 2.03$, $P = 0.051$).

Relationship between pollen deposition and style curvature

We included both linear and quadratic terms for pollen number as independent variables to account for the curvilinearity of the response variable, the degree of style curvature (Neter *et al.* 1996). This quadratic relationship between the amount of pollen deposited on the stigma and the degree of style curvature was highly significant (regression, $F_{2,37} = 230.93$, $P < 0.001$; coefficients \pm s.e., linear term: 0.182 ± 0.013 , $t = 13.67$, $P < 0.001$; quadratic term: -0.001 ± 0.0001 , $t = 8.20$, $P < 0.001$; $n = 40$ styles; Fig. 3). Overall, style curvature was prevented when 50 or more grains were deposited per stigma.

Discussion

Our data show that *H. trionum* var. *vesicarius* is fully self-compatible and capable of delayed autonomous self-pollination. However, seed set of some plants was low following autonomous selfing, indicating that pollinators are often required for maximum seed production. Although seed set following self- or cross-fertilisation did not differ, outcrossed progeny had higher fitness than selfed progeny,

with inbreeding depression being expressed late in the life cycle. Under natural conditions plants probably produce a mixture of selfed and crossed seeds, the frequency of each depending on pollinator abundance and visitation. Thus, delayed selfing permits plants to exploit the 'best of both worlds' and assists in weathering the vagaries of pollination within and among years (Becerra and Lloyd 1992; Kalisz and Vogler 2003).

While prior or competing autonomous selfing is expected to evolve when pollinators are in chronic short supply, selection for delayed selfing is predicted when pollinator visitation is variable (Schoen and Brown 1991; Lloyd 1992). Delayed selfing allows outcrossing to occur when pollinators are available, but ensures reproduction and increases plant fitness if pollinators fail to visit flowers, providing that some selfed progeny survive to maturity (Kalisz and Vogler 2003; Kalisz *et al.* 2004). Populations of *H. trionum* var. *vesicarius* occur in dry areas of inland Australia, where rainfall can be erratic. Plants quickly appear after heavy rains and can complete their life cycle in about 8 weeks, depending on soil moisture conditions. In such habitats, pollinators are likely to vary in space and time, rendering cross-pollination unpredictable. Floral densities may also vary, potentially exaggerating the effect of low pollinator abundance through competition among flowers for pollinators (Fritz and Nilsson 1994). Seed (2003) found that when *H. trionum* var. *vesicarius* flowers were closing, 70% of styles had curved, indicating that they would be autonomously self-pollinated to some extent. The remaining 30% of flowers had straight styles and heavy pollen loads that probably included some cross-pollen deposited by native bees and introduced honeybees. Under such pollination conditions, mixed mating would be expected. Genetic studies are now required to assess the amount of self- and cross-fertilisation occurring in populations, and how plant mating relates to the pollinator environment.

Cumulative inbreeding depression averaged 0.64. Because we assessed relative fitness under favourable conditions in the glasshouse, inbreeding depression may be higher under natural field conditions (Ramsey and Vaughton 1998). Our estimate of inbreeding depression is significantly greater than 0.5, the theoretical value of inbreeding depression above which the automatic gene transmission advantage conveyed by self-fertilisation is negated (Lande and Schemske 1985; Charlesworth and Charlesworth 1987). Simple models predict that, unlike prior and competing selfing, delayed selfing can evolve in spite of strong inbreeding depression. Delayed selfing can provide reproductive assurance and increase fitness by fertilising ovules that would otherwise remain unfertilised, leading to stable mixed mating (Lloyd 1979, 1992; Kalisz *et al.* 2004). By contrast, prior and competing selfing usurp ovules that could potentially be outcrossed, leading to genetic costs of reproductive assurance (i.e. seed discounting, Lloyd 1992). However, seed discounting could also be associated with delayed

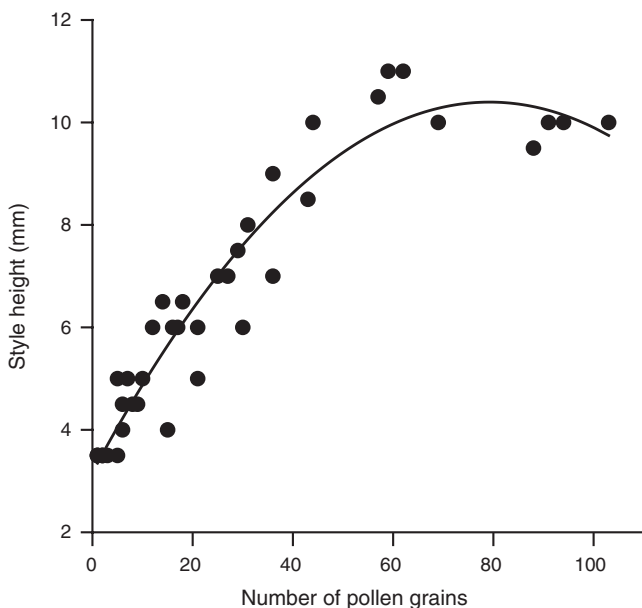


Fig. 3. Quadratic relationship between style height and the number of pollen grains per stigma in *Hibiscus trionum* var. *vesicarius*. Larger values for height indicate minimum curvature of the style branches, whereas smaller values indicate substantial curvature ($y = 3.27 + 0.182x - 0.001x^2$, $r^2 = 0.93$, $F_{2,37} = 230.93$, $P < 0.001$, $n = 40$ styles).

selfing, if inbreeding depression is high and selfed seeds pre-empt resources that could have been used to produce more or better provisioned crossed seeds (Klüber and Eckert 2004). To assess the adaptive benefit of delayed selfing in providing reproductive assurance in *H. trionum* var. *vesicarius*, future work needs to address the occurrence and potential fitness costs of seed discounting as Eckert and co-workers have done for *Aquilegia canadensis* (reviewed by Eckert and Herlihy 2004).

The magnitude of inbreeding depression in *H. trionum* var. *vesicarius* was ~4-fold greater than in *H. trionum* var. *trionum* ($\delta = 0.15$, Ramsey *et al.* 2003). Plants of both varieties were grown under similar conditions and the disparate estimates probably reflect differences in their mating histories. The magnitude of inbreeding depression is often related to the selfing rate in populations because greater self-fertilisation provides opportunities for purging of deleterious alleles and reduces genetic load (Byers and Waller 1999). The introduced *H. trionum* var. *trionum* is a weed of cultivated and disturbed sites and has probably experienced greater selfing after plants were introduced in Australia than the native *H. trionum* var. *vesicarius*. Inbreeding depression in *H. trionum* var. *vesicarius* is similar to that found in predominantly outcrossing species ($\delta = 0.53$), but is expressed at later life-cycle stages, as is typically found in selfing species (reviewed by Husband and Schemske 1996).

Seed set following delayed autonomous selfing in *H. trionum* var. *vesicarius* varied among maternal plants, ranging from 11 to 103% that of hand-selfing. Low autonomous seed set in some plants did not result from variation in self-fertility or the failure of styles to curve. Instead, the variation was related to the position of anthers on the staminal column, such that stigmas only partially contacted the anthers as flowers closed. In *H. laevis*, the ability of plants to self autonomously is also related to differences in the separation of stigmas and anthers (i.e. herkogamy, Klips and Snow 1997); however, herkogamy in our study species is much less pronounced. Variation in autonomous selfing in *H. trionum* var. *vesicarius* contrasts with results for *H. trionum* var. *trionum*, where autonomous seed set was consistently high for all plants (Ramsey *et al.* 2003). More efficient autonomous selfing in *H. trionum* var. *trionum* is likely to reflect stronger selection for reproductive assurance. The strength of selection for reproductive assurance will be determined by the variation in pollination uncertainty and any negative effects of delayed selfing, such as seed discounting. Depending on the relative magnitude of these factors, the frequency of fully autonomous genotypes could change over generations, thus altering the mean autonomous-selfing rate of populations (Bixby and Levin 1996).

Delayed selfing in *H. trionum* var. *vesicarius* was caused by the styles curving and the stigmas contacting the anthers as the flowers closed. However, the delayed selfing was facultative, and style curvature could be prevented or greatly

reduced if stigmas were pollinated with fresh pollen. The degree of curvature depended on the number of pollen grains on the stigma, with 50 or more grains preventing curvature and autonomous selfing. Facultative delayed selfing has been rarely reported, and in *Hibiscus* was first found in an unspecified variety of *H. trionum* by Buttrose *et al.* (1977). More recently, facultative delayed selfing has been reported in *H. laevis* (Klips and Snow 1997). Interestingly, unlike our findings for *H. trionum* var. *vesicarius*, pollinated styles of *H. laevis* still curve, although to a lesser extent than unpollinated styles (see fig. 4 in Klips and Snow 1997). This may indicate that the mechanism preventing style curvature is better developed in *H. trionum* var. *vesicarius* than in *H. laevis*.

The significance of facultative style curvature is unclear. Buttrose *et al.* (1977) interpreted facultative style curvature as a mechanism to promote outcrossing by preventing the dilution of cross-pollen by self-pollen after pollinators visited flowers. By contrast, Klips and Snow (1997) argued that facultative style curvature would have little effect on the mating system in *H. laevis*, because early arriving cross-pollen would germinate and fertilise ovules before delayed selfing occurred. They proposed that reduced curvature of pollinated styles was probably caused by pollen-tube growth that stiffened the styles, rendering curvature difficult. We found that deposition of live pollen on stigmas, but not dead pollen, prevented curvature. This indicates that either pollen germination and possible hormonal signals to transmitting tissues or pollen-tube growth, as proposed by Klips and Snow (1997), prevents style curvature. We also found that both self- and cross-pollination prevented style curvature, indicating that pollen genotype does not influence the behaviour of styles. Thus, under natural conditions either outcrossing or selfing mediated by pollinators (i.e. geitonogamy and autogamy) would prevent style curvature.

When we pollinated different numbers of styles within a flower, the remaining unpollinated styles curved, indicating that the individual styles acted independently of each other. Moreover, when we pollinated only one style per flower, seed set was only ~50% that of flowers for which all five stigmas were pollinated. Thus, style independence within flowers would ensure that if cross-pollination was insufficient to fertilise all ovules, then autonomous selfing would augment overall seed set, and result in a mixture of crossed and selfed seeds within fruits. In contrast to our results, Buttrose *et al.* (1977) found in *H. trionum* that styles were not independent. If only one style was pollinated, then curvature of the other styles within a flower was prevented. This lack of style independence could be disadvantageous under conditions of limited cross-pollination, if, as occurs in *H. trionum* var. *vesicarius*, pollination of only one style results in fewer seeds being produced than when all of the styles are pollinated.

For *H. trionum* var. *vesicarius*, delayed selfing would provide considerable mating flexibility and has probably contributed to the persistence of populations in their natural

habitat where pollinators are unpredictable. As suggested by Kalisz and Vogler (2003), more studies are required to assess the proportion of species that utilise autonomous delayed selfing, and to investigate the ecological context favouring mixed mating. We are currently investigating the role of delayed selfing in providing reproductive assurance under natural conditions to better explain the breeding system of *H. trionum*.

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