

An experimental evaluation of self-interference in *Narcissus assoanus*: functional and evolutionary implications

A. C. CESARO,* S. C. H. BARRETT,† S. MAURICE,‡ B. E. VAISSIERE§ & J. D. THOMPSON*

*Centre d'Ecologie Fonctionnelle et Evolutive, CNRS, Montpellier Cedex 5, France

†Department of Botany, University of Toronto, Toronto, Ontario, Canada

‡I.S.E.M, University of Montpellier II, Montpellier Cedex 5, France

§Laboratoire de Pollinisation Entomophile, UMR 406 Ecologie des Invertébrés, INRA site Agroparc, Avignon, France

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Abstract

Floral traits that reduce self-pollination in hermaphroditic plants have usually been interpreted as mechanisms that limit the genetic consequences of self-fertilization. However, the avoidance of sexual conflict between female and male function (self-interference) may also represent an important selection pressure for the evolution of floral traits, particularly in self-incompatible species. Here, we use experimental manipulations to investigate self-interference in *Narcissus assoanus*, a self-incompatible species with a stigma-height dimorphism in which the degree of spatial separation between sex organs (herkogamy) differs strikingly between the long- and short-styled morphs (hereafter L- and S-morphs). We predicted that weak herkogamy in the L-morph would cause greater self-pollination and hence self-interference. Experimental self-pollination reduced seed set when it occurred prior to, or simultaneously with, cross-pollination in the L-morph, but only if it occurred prior to cross-pollination in the S-morph. In the field, autonomous self-pollination was greater in the L-morph than the S-morph, but we found no evidence that self-interference reduced maternal or paternal fitness in either morph. One-day-old flowers of the L-morph have reduced stigma receptivity and hence exhibit protandry, whereas stigma receptivity and anther dehiscence are concurrent in the S-morph. This suggests that the two style morphs have alternative strategies for reducing self-interference: dichogamy in the L-morph and herkogamy in the S-morph. These results provide insight into the mechanisms that reduce sexual conflict in hermaphrodite plants and are of significance for understanding the evolution and maintenance of sexual polymorphisms.

Introduction

Hermaphroditism is the predominant sexual condition in flowering plants with most species possessing perfect (bisexual) flowers. The presence of female and male sex organs within individual flowers has the advantage of economizing on the resources allocated to pollinator attraction and in facilitating selfing when mates or pollinators are scarce (Charnov, 1982; Charlesworth &

Charlesworth, 1987a; Lloyd, 1987). However, hermaphroditism can be disadvantageous if self-pollination results in selfing and inbreeding depression. Indeed, floral traits that reduce the incidence of self-pollination have usually been interpreted as 'anti-selfing mechanisms' as they limit the genetic costs resulting from inbreeding depression (Darwin, 1876; Charlesworth & Charlesworth, 1987b). However, many plant species are protected from the harmful effects of selfing and inbreeding depression by physiological self-incompatibility yet they still possess floral traits interpreted as 'anti-selfing mechanisms'. This potential redundancy of function has recently stimulated efforts to find alternative adaptive explanations for a

Correspondence: A. C. Cesaro, Centre d'Ecologie Fonctionnelle et Evolutive, CNRS, 1919 Route de Mende, 34293, Montpellier Cedex 5, France. Tel.: +34 676 13214; Fax: +34 674 12138; e-mail: Thompson@cefe.cnrs-mop.fr

diversity of floral traits (Harder & Barrett, 1996; Harder *et al.*, 2000). For example, it has been proposed that the spatial separation of stigmas and anthers (herkogamy), or the temporal separation of stigma receptivity and anther dehiscence (dichogamy) may largely function to avoid interference between pollen dispersal and receipt (Lloyd & Webb, 1986; Webb & Lloyd, 1986). According to this hypothesis, these traits reduce sexual conflicts that arise because of the fundamental hermaphrodite condition of most flowering plants. Lloyd & Webb (1986) proposed that self-interference is one of the most important selective forces shaping floral evolution.

Self-interference may potentially compromise both maternal and/or paternal fitness (Lloyd & Yates, 1982; Lloyd & Webb, 1986; Webb & Lloyd, 1986; Bertin, 1993; Bertin & Newman, 1993; Harder & Barrett, 1995; Harder *et al.*, 2000; Barrett, 2002a,b). Female fitness may be negatively affected in three main ways. First, the presence of stamens or the process of pollen removal from anthers may obstruct pollen deposition on stigmas (Barrett & Glover, 1985; Palmer *et al.*, 1989). Secondly, the deposition of self-pollen may cause stigma or stylar clogging and reduce the opportunities for the subsequent growth of pollen tubes (Bawa & Opler, 1975; Howlett *et al.*, 1975; van der Pijl, 1978; Shore & Barrett, 1984; Scribailo & Barrett, 1991; Scribailo & Barrett, 1994). Thirdly, in self-compatible species or in species with late-acting self-incompatibility, self-pollen tubes may usurp or disable ovules which are then unavailable for cross-fertilization, i.e. ovule discounting (Bertin & Sullivan, 1988; Waser & Price, 1991; Barrett *et al.*, 1996; Sage *et al.*, 1999). There are two main ways that male fitness may be reduced because of self-interference, either by reduced pollen dispersal, i.e. if styles and stigmas physically obstruct access to anthers (Baker, 1964; Lloyd & Yates, 1982; Kohn & Barrett, 1992; Fetscher, 2001), or as a result of pollen discounting, i.e. the reduction in the availability of pollen for outcrossing (Harder & Schemske, 1995; Harder & Wilson, 1998). In contrast to information on the genetic costs of selfing in plants (reviewed in Charlesworth & Charlesworth, 1987b; Husband & Schemske, 1996), the relative importance of these different forms of interference and their significance for floral evolution is still poorly understood.

Species that exhibit variation in herkogamy can provide useful experimental systems for studying the reproductive consequences of self-interference. For example, in approximately a dozen *Narcissus* species populations are commonly dimorphic for style length with the style morphs differing significantly in their degree of herkogamy (Barrett *et al.*, 1996; Baker *et al.*, 2000a; Arroyo *et al.*, 2002). In the L-morph, stigmas are usually positioned slightly above or at the same position as the anthers, whereas in the S-morph, stigmas are positioned well below the anthers. The weak herkogamy of the L-morph might be expected to cause greater levels of self-pollination in comparison with the S-morph.

Previous experimental pollination studies on *Narcissus* species, e.g. *N. tazetta* (Dulberger, 1964) and *N. triandrus* (Sage *et al.*, 1999) have demonstrated that self-pollination causes considerable ovule sterility as a result of the operation of a late-acting ovarian self-incompatibility system. Where this occurs prior self-pollination could potentially limit outcrossed seed set as a result of ovule discounting (Dulberger, 1964; Barrett *et al.*, 1996; Sage *et al.*, 1999; Arroyo *et al.*, 2002). The extent to which the difference in herkogamy between the style morphs in *Narcissus* species influences their susceptibility to self-interference has not been examined.

Here we investigate the reproductive consequences of self-interference in the partially self-incompatible perennial geophyte *Narcissus assoanus*. This species has well-developed stigma-height dimorphism and a striking difference in herkogamy between the two style morphs (see Baker *et al.*, 2000a for details). We begin by examining aspects of the floral biology of *N. assoanus* that are likely to influence self-interference. Specifically, we investigate temporal aspects of female and male function in the style morphs, including the timing of stigma receptivity and anther dehiscence, the duration of pollen viability and the patterns of pollen-tube growth in self- and cross-pollinations. Through controlled pollination studies, we then determine whether prior self-pollination influences outcrossed seed set. Having established the potential for self-interference in *N. assoanus*, we then evaluate under field conditions if the levels of self-pollination differ between the style morphs and assess the extent to which self-interference reduces female fertility and pollen transfer. Finally, we discuss the role of self-interference in the evolution and maintenance of stigma-height dimorphism and incorporate our results into models developed previously to identify the stability of this polymorphism.

Materials and methods

Study organism and sites

Narcissus assoanus is an insect-pollinated geophyte common in lowland garrigues and upland grassland on limestone in the Iberian Peninsula and southern France. Flowering plants usually produce one, or less often two, yellow flowers with a prominent bowl-shaped corona and a long narrow floral tube. All plants we used in our study had one flower. The spatial separation between the closest anthers and the stigma in the L-morph (mean = 0.4 mm) is significantly less than in the S-morph (mean = 4.9 mm) in the population used in this study. The two style morphs do not differ in pollen production or pollen size (Baker *et al.*, 2000a). Controlled self- and cross-pollination indicate that *N. assoanus* is moderately self-sterile (8% seed set from selfing) and fully cross-compatible regardless of morph identity (Baker *et al.*, 2000b). Pollination is primarily by Cleopatra butterflies

(*Gonepteryx cleopatra*), hawkmoths (*Macroglossum stellatarum*) and solitary bees (*Anthophora* spp.) (Baker *et al.*, 2000c).

We sampled the plants used in this study from a natural population near Blandas on an upland limestone plateau 60 km northwest of Montpellier in southern France. We collected bulbs from the field prior to flowering in early spring 2002 and 2003 and transferred them to a glasshouse at the CEFÉ-CNRS experimental garden in Montpellier. We separated the bulbs into 12 groups depending on their spatial location in the field. In our study, all cross-pollinations (see below) were made between plants from different groups to minimize biparental inbreeding and to completely avoid self-pollination (bulb-splitting is common at this site: J.D. Thompson and L.D. Harder, unpublished data). Hand-pollinations were conducted on partially emasculated flowers (upper anthers were emasculated on all flowers to prevent selfing in the L-morph) with a large amount of one-donor pollen brushed across the full extent of the stigma.

We conducted the field studies described below in four populations: the natural population near Blandas, a second population approximately 2 km distant near the village of Rogues and two other populations near the villages of Ceyrac and Pompignan in lowland garrigues, both approximately 40 km north of Montpellier. Ceyrac and Pompignan are approximately 50 km from Blandas. In each population, the L- and S-morphs are equally present. All experimental work was conducted in April 2002 and March to April 2003.

Floral biology

Stigma receptivity

To determine the timing of stigma receptivity, we compared the adherence of self- and cross-pollen with stigmas of different ages, i.e. 1, 2 and 3-day-old flowers of each morph in April 2003. We used 14 plants of each morph in each of six combinations of two types of pollination and three flower ages (i.e. a total of 168 plants). Styles were cut 1 h after hand-pollination. For each plant, we counted the number of pollen grains that had adhered to the stigma (some of which had started to germinate) under a light microscope. We stained pollen grains with basic fuchsin to permit accurate counting. To determine if variation in stigma receptivity influenced seed set we conducted hand-pollinations in the glasshouse with cross-pollen on 1, 2 and 3-day-old flowers on 10 plants of each morph in April 2002 (60 flowers in total). Fruit were left to mature and seed were counted.

Anther dehiscence and pollen viability

To determine the timing of anther dehiscence, we collected 140 bulbs (70 of each morph) from the Blandas population in early April 2003. Anther dehiscence was monitored outdoors in the CEFÉ-CNRS experimental garden in Montpellier in newly opened flowers and 12,

24, 48, 96 h after flower opening. For each flower, we classified anthers into one of five classes: (i) both anther levels undehisced, (ii) top of the upper anthers dehiscenced, lower anthers undehisced, (iii) upper anthers dehiscenced completely, lower anthers undehisced, (iv) upper anthers and top of lower anthers dehiscenced, and (v) upper anthers and lower anthers completely dehiscenced. We never observed a case of upper anthers undehisced and lower anthers dehiscenced.

To estimate pollen viability, we conducted hand-pollination in the glasshouse on 3-day-old emasculated flowers of the style morphs using pollen from flowers that were 1, 2, 3, 4, or 8 days old. We used 14 plants of each donor morph in each treatment (a total of 140 plants). All pollinations involved crosses among plants of the alternate morph.

Pollen-tube growth

To assess pollen-tube growth in styles, we conducted outcross- and self-pollinations in the glasshouse in April 2002. We collected flowers 12, 24, 46 or 96 h after pollination, and immediately fixed and prepared them for fluorescence microscopy as described by Williams & Knox (1982). For cross-pollinations, individual plants were crossed with a single pollen donor of the alternate morph. Ten plants of each morph were used in each of the four time collections and two pollination treatments, for a total of 80 plants per morph in the experiment. At each harvest time, we measured style length and maximum pollen-tube length to determine whether there were differences between the style morphs in pollen-tube growth after cross- or self-pollination.

Self-interference under experimental and natural conditions

Glasshouse pollinations

To evaluate the influence of self-pollination on the seed set of cross-pollinated flowers, six hand-pollination treatments were performed in the glasshouse in April 2002: (i) cross-pollination, (ii) simultaneous self- and cross-pollination (iii) self-pollination followed by cross-pollination 12 h later, (iv) self-pollination followed by cross-pollination 24 h later, (v) self-pollination followed by cross-pollination 48 h later, and (vi) self-pollination. Fifteen plants of each morph were used in each of the six treatments for a total of 180 plants.

Self-pollination in the field

We estimated self-pollen deposition in natural conditions at the Blandas population by randomly marking 286 flower buds with coloured tape at the base of the floral stem between the 5 and 17 April 2002. We randomly allocated buds to one of three treatments: (i) unmanipulated flowers, to assess total (self + outcross) pollen deposition in natural conditions ($n = 34, 32$ for L- and S-morphs), (ii) flowers emasculated prior to opening, so

they only received outcross pollen ($n = 30, 34$ for L- and S-morphs), and (iii) flowers caged, and thus only autonomous self-pollination ($n = 58, 70$ for L- and S-morphs). Flowers were collected 7–8 days later. For each plant, we counted the number of pollen grains on the stigma (stained with fuchsin) using a light microscope.

Self-interference in the field

To investigate whether there were any costs of self-interference in the wild we conducted two treatments at the Pompignan (20 to 30 March 2003) and Blandas (6 to 16 April 2003) sites: (i) unmanipulated flowers with natural seed-set (Pompignan: $n = 16, 16$ for L- and S-morphs; Blandas: $n = 24, 22$ for L- and S-morphs), (ii) emasculated flowers which could not experience self pollen interference as they only received outcross pollen (Pompignan: $n = 20, 13$ for L- and S-morphs; Blandas: $n = 20$, for both the L- and S-morphs). These sites were chosen because they experience contrasting rates of pollinator visitation, with much higher visitation in the Pompignan site than in the Blandas site (J.D. Thompson and A. Cesaro, unpublished data).

To investigate whether pollen removal and transfer is obstructed by the presence of the style in the L-morph, we randomly set out six replicate plots of each of two treatments in part of the Ceyrac population where large numbers of flower buds were emerging in March 2003. We monitored all new flowers in each plot to create two treatments that differ only in the morphology of the pollen donor, i.e. (i) plots with L-morph flowers as pollen donors and emasculated S-morph flowers as recipients, (ii) plots with L-morph donors that had their style excised and emasculated S-morphs as recipients. In each plot we maintained a ratio of two donors for each recipient. The area of each plot was a little $<1 \text{ m}^2$ with a 1 m border in which all flowers buds were removed during the course of the experiment.

In a previous study at this site using similar sized and spaced plots, Thompson *et al.* (2003) detected significant variation in seed set among plots that differed in morph frequency. This indicates that pollen delivery to stigmas from outside plots does not mask effects related to differential pollen transfer within experimental treatments. In a parallel study at this site (A. Cesaro & J. Thompson, unpublished data) we also set out two plots containing only emasculated flowers to assess pollen flow into plots. In these control plots, seed per flower ratio (mean = 0.09 ± 0.02), fruit per flower ratio (mean = 0.36 ± 0.13) and seed per fruit ratio (mean = 0.25 ± 0.02) were much lower than plots with pollen donors present. These observations indicate that any fertility differences between treatments that we detect in this study are likely to be associated with our experimental manipulations.

From 19 to 28 March, we visited all plots daily and from 29 March to 3 April, every 2 days before 10 h to maintain the experimental plots. During each visit we

marked all experimental flowers at the base of the stem with sticky tape to identify donors and recipients and to check for newly opened flowers. The experiment involved a total of 366 plants. On 15 May, we harvested all fruits to assess fruit-set, seed-set and ovule number. All flowers or fruits suffering herbivory were excluded from the experiment.

Statistical analyses

All statistical analyses were conducted using SAS 2001. In the experiment concerning stigma adherence, we performed a three-way ANOVA using PROC GLM with flower age, type of cross and morph as fixed effects. In the experiment concerning self-pollen deposition, we performed a two-way ANOVA using PROC GLM with treatment and morph as fixed effects. Our analysis of pollen-tube growth involved a three-way ANOVA (PROC GLM) with type of pollination (self vs. cross), time period and morph as fixed effects. For the timing of anther dehiscence, the number of plants of each morph in each class was analysed using generalized linear models (PROC GENMOD) with a binomial error distribution (McCullagh & Nelder, 1989). For all other treatments, we analysed data for fruit-set per flower and seed-set per ovule using PROC GENMOD. Analyses were conducted in three ways: (i) on the full data set, (ii) by morph to assess treatment effects on each morph and (iii) by treatment to assess where morph-specific differences occurred.

Results

Temporal aspects of female and male function

Stigma receptivity

We found 76% fewer pollen grains on 1-day-old stigmas of the L-morph relative to all other stigmas (older L-morph or S-morph) (Fig. 1). Hence, a significant effect

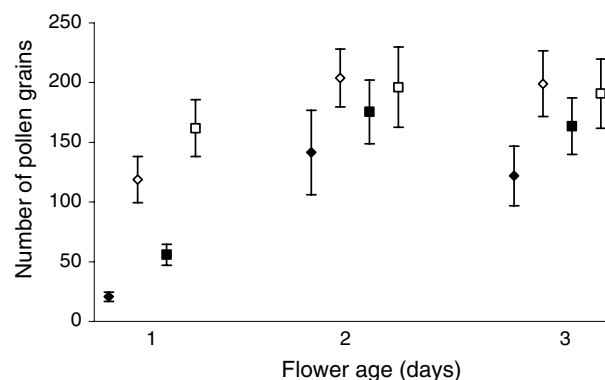


Fig. 1 Pollen grain number (mean \pm SE) on stigmas of the L-morph (filled shapes) and S-morph (open shapes) of *Narcissus assoanus* after self- (diamonds) and cross- (squares) pollinations on 1, 2 and 3-day-old flowers.

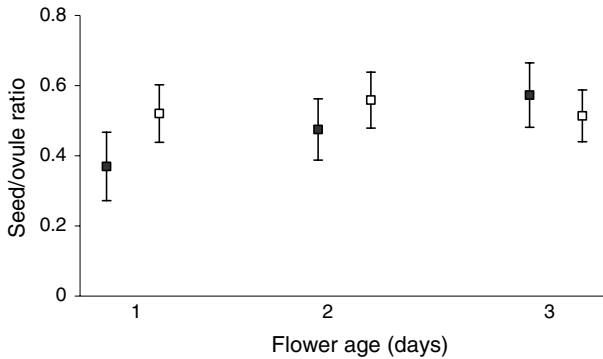


Fig. 2 Mean (\pm SE) proportion seed set per flower for the L-morph (■) and the S-morph (□) in hand-pollinated flowers of different ages in *Narcissus assoanus* under glasshouse conditions.

of flower age on pollen adherence to stigmas was detected for the L-morph ($F_{2,54} = 15.88$, $P < 0.001$) but not for the S-morph ($F_{2,54} = 3.10$, n.s.). There were significant differences between morphs ($F_{1,108} = 20.59$, $P < 0.001$) in the number of pollen grains on stigmas because of greater adherence of pollen on stigmas of the S-morph (Fig. 1) but no significant effect of cross type ($F_{1,108} = 2.54$, n.s.) and no significant interactions between age and cross type ($F_{2,108} = 0.32$, n.s.), between morph and cross type ($F_{1,108} = 0.93$, n.s.), between morph and flower age ($F_{2,108} = 1.68$, n.s.) or the age by morph by cross type interaction ($F_{2,108} = 0.39$, n.s.).

There was no effect of flower age on seed set following controlled cross-pollination in the S-morph ($G = 0.01$, d.f. = 2, n.s.). In contrast, in the L-morph seed set in one and 2-day-old flowers was reduced by 22% ($G = 13.51$, d.f. = 2, $P < 0.01$; Fig. 2). Therefore seed set exhibited a significant morph by treatment interaction ($G = 7.30$, d.f. = 2, $P < 0.05$) and a significant effect of flower age ($G = 6.76$, d.f. = 2, $P < 0.05$) but no significant difference between morphs ($G = 0.46$, d.f. = 1, n.s.). For fruit set (mean = 0.87 ± 0.1) there was no significant effect of treatment ($G = 2.27$, d.f. = 2, n.s.), morph ($G = 1.88$, d.f. = 1, n.s.), or morph by treatment interaction ($G = 1.18$, d.f. = 2, n.s.).

Anther dehiscence and pollen viability

Anther dehiscence proceeded from the top to the bottom of individual anthers and from the upper to the lower anthers. Upper anthers dehiscid just before flowers opened and there was no significant difference in this sequence between morphs ($F = 0.01$, d.f. = 1, n.s.).

There was significant variation in cross-pollinated seed set among donor flowers with pollen of different ages ($G = 53.61$, d.f. = 4, $P < 0.001$), between morph ($G = 13.43$, d.f. = 1, $P < 0.001$) and because of a morph by treatment interaction ($G = 11.86$, d.f. = 4, $P < 0.05$). There was no significant difference in seed set using pollen from flowers <4 days old whereas seed set using

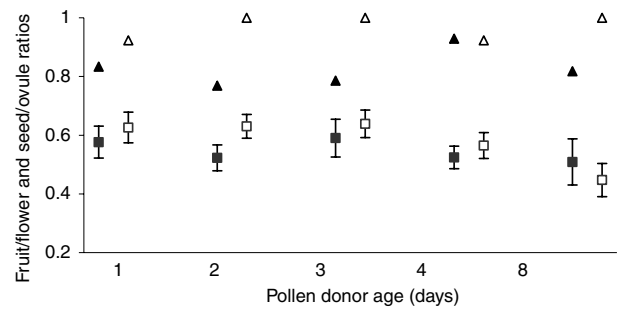


Fig. 3 Mean (\pm SE) fruit-flower ratio (triangles) and seed-ovule ratio per fruit (squares) after inter-morph pollinations using S-morph (filled shapes) and L-morph (open shapes) pollen donors of different ages.

8-day-old pollen was reduced by 28% with L-morph donors and 10% with S-morph donors (Fig. 3). Pollen from both morphs showed a significant effect of flower age (L-morph: $G = 12.18$, d.f. = 4, $P < 0.05$; S-morph: $G = 60.94$, d.f. = 4, $P < 0.001$). Fruit-set did not vary significantly among pollen donors of different age ($G = 0.54$, d.f. = 4, n.s.) but did vary significantly between pollen donors of each morph ($G = 7.86$, d.f. = 1, $P < 0.01$).

Pollen-tube growth

Pollen grains germinated within 12 h after pollination and pollen tubes grew to similar lengths after self- and cross-pollination regardless of the time since pollination ($F_{1,133} = 0.39$, n.s.; Fig. 4). After 4 days, the majority of self- and cross-pollinated styles of both morphs had large numbers of pollen tubes at the base of the style, entering the ovary. Because L-morph styles are longer than S-morph styles, we found a significant effect of morph ($F_{1,133} = 186.20$, $P < 0.001$) and a significant morph by time period interaction ($F_{3,133} = 24.01$, $P < 0.001$) on pollen-tube length. Pollen tubes reached the ovary

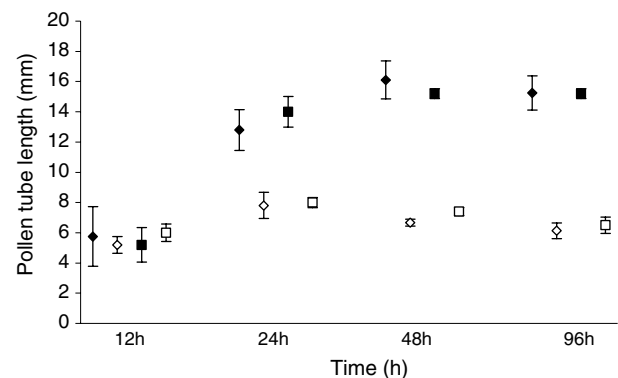


Fig. 4 The length of pollen tubes after outcross (squares) and self-pollination (diamonds) in the L- (filled shapes) and S-morph (open shapes) of *Narcissus assoanus* under glasshouse conditions.

12–24 h after pollination in the S-morph and after 24–48 h in the L-morph.

Self-interference under controlled and natural conditions

Glasshouse experiment

In both morphs, self-pollination 12, 24 and 48 h prior to cross-pollination significantly reduced seed set. Indeed, self-pollination 24 h or more before cross-pollination reduced seed set to values similar to those after self-pollination (Fig. 5). Seed set showed significant variation among treatments ($G = 411.69$, d.f. = 5, $P < 0.001$) and between morphs ($G = 15.12$, d.f. = 1, $P < 0.001$) and a significant morph by treatment interaction ($G = 12.47$, d.f. = 5, $P < 0.05$). This interaction indicates that although both morphs showed a significant effect of treatment (L-morph: $G = 188.20$, d.f. = 5, $P < 0.001$; S-morph: $G = 240.19$, d.f. = 5, $P < 0.001$), the response to the treatments was not the same in the two morphs. For the L-morph, seed set following cross-pollination was significantly greater than all treatments in which self-pollination occurred, and even simultaneous self- and cross-pollination caused reduced seed set. In contrast, self-pollination at the same time as outcross pollination in the S-morph did not result in a significant reduction of seed set relative to cross-pollination. In the S-morph, self-pollination had a negative effect on seed set only if it occurred prior to cross-pollination. Fruit-set did not vary significantly between treatments ($G = 2.54$, d.f. = 5, n.s.) or morphs ($G = 0$, d.f. = 1, n.s.) and did not show a significant morph by treatment interaction ($G = 0.65$, d.f. = 5, n.s.).

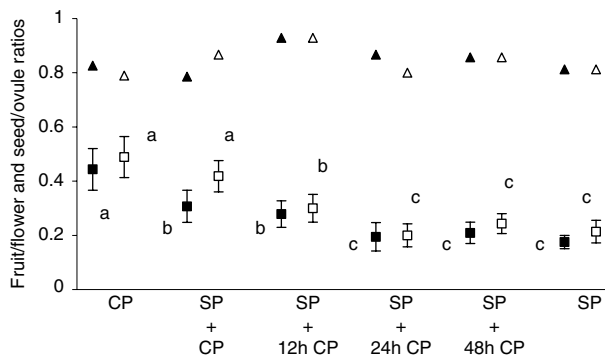


Fig. 5 The influence of self-pollination and cross-pollination on fruit-flower ratio (triangles) and seed-ovule ratio per fruit (squares) (mean \pm SE) in the L- (filled shapes) and S-morphs (open shapes) of *Narcissus assoanus*. The six experimental treatments are: (CP) cross-pollination, (SP + CP) self-pollination + cross-pollination at the same time, (SP + 12 h CP) self-pollination + cross-pollination after 12 h, (SP + 24 h CP) self-pollination + cross-pollination after 24 h, (SP + 48 h CP) self-pollination + cross-pollination after 48 h, and (SP) self-pollination. Mean values are significantly different if they have different letters (a, b, c).

Field experiments

As predicted, autonomous self-pollen deposition was greater in the L-morph compared with the S-morph (Fig. 6). We recorded significantly more pollen grains on stigmas of the L-morph than on stigmas of the S-morph in caged flowers ($F_{1,128} = 10.59$, $P < 0.01$), but not on unmanipulated ($F_{1,48} = 0.76$, n.s.) or emasculated ($F_{1,20} = 0.07$, n.s.) flowers following insect visitation. There was no difference between the unmanipulated and the emasculated treatments ($F_{1,68} = 1.78$, n.s.). Overall, the number of pollen grains on stigmas showed no significant differences between morphs ($F_{1,196} = 0.79$, n.s.) and no significant morph by treatment interaction ($F_{2,196} = 1.47$, n.s.). Both morphs showed a significant effect of treatment (L-morph: $F_{2,98} = 31.05$, $P < 0.001$; S-morph: $F_{2,98} = 11.40$, $P < 0.001$).

The presence of anthers and thus self-pollen did not reduce seed set of the L- or S-morph under natural conditions. Mean fruit set was approximately 0.84 in both sites and seed set (which only varied from 0.32 to 0.38 in the eight combinations of treatment, morph and site) did not vary significantly between emasculated and non-emasculated flowers ($G = 1.08$, d.f. = 1, n.s.; $G = 3.47$, d.f. = 1, $P > 0.05$), between morphs ($G = 2.48$, d.f. = 1, n.s.; $G = 3.72$, d.f. = 1, n.s.) or between populations ($G = 0.01$, d.f. = 1, n.s.; $G = 0.13$, d.f. = 1, n.s.). Observations of pollinators indicated that flowers with and without anthers were visited similarly, probably as the main pollinators feed on nectar.

We found no evidence that the style obstructs the ability of the L-morph to transfer pollen to neighbouring plants of the S-morph. The S-morph exhibited no significant variation in either fruit-set ($G = 0.15$, d.f. = 1, n.s.) or seed-set ($G = 1.69$, d.f. = 1, n.s.) between plots with unmanipulated donors of the L-morph (fruit-set = $0.91 \pm \text{SE} = 0.04$; seed-set = $0.51 \pm \text{SE} = 0.03$) or L-morph donors with excised styles (fruit-set = $0.87 \pm \text{SE} = 0.03$; seed-set = $0.52 \pm \text{SE} = 0.03$).

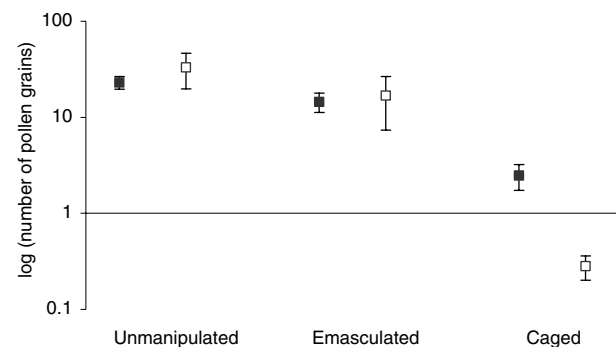


Fig. 6 The mean number (on a log scale) of pollen grains (\pm SE) on stigmas of the L- (■) and S-morph (□) in unmanipulated (open-pollinated), emasculated and caged plants of *Narcissus assoanus* at the Blandas site.

Discussion

Our study illustrates how floral traits may serve to limit the costs of self-interference in hermaphrodite plants. As the species (*N. assoanus*) we investigated is self-incompatible the floral traits that distinguish the style morphs are likely to function to promote more effective cross-pollination by reducing gamete wastage. Indeed, recent experimental studies of this species support this interpretation (Thompson *et al.*, 2003). Here we provide evidence that the style morphs of *N. assoanus* exhibit different floral strategies (herkogamy vs. dichogamy) for reducing self-pollination. To our knowledge this is the first demonstration of the occurrence of a polymorphism for traits that limit sexual conflict in plants. In our discussion we consider how morph-specific traits function to reduce self-interference and the role that ovule discounting may play in the evolution and maintenance of sexual polymorphisms in *Narcissus*.

Morph-specific patterns in costs and avoidance of self-interference

Our experiments indicate that prior self-pollination has negative consequences for outcrossed seed-set under controlled glasshouse conditions (Fig. 5). Simultaneous self- and cross-pollination reduced seed-set by 20% in comparison with cross-pollination alone. Self-pollination followed 24 h later by cross-pollination decreased seed set by 60% to a level equivalent to that obtained by selfing. In the related *N. triandrus*, self-pollination 24 h prior to cross-pollination can reduce maternal fitness by up to 75% because of reduction in the availability of fertile ovules following embryo sac degeneration after self-pollination (Barrett *et al.*, 1997; Sage *et al.*, 1999). As *N. assoanus* self-pollen tubes regularly reach the ovules, it seems very likely that a similar mechanism occurs in this species. The maternal cost of self-pollination we detected is thus likely to be because of ovule discounting resulting from embryo sac degeneration after self-pollination.

An important finding of our study is that autonomous self-pollination is substantially higher in the L-morph than in the S-morph (Fig. 6). Whereas the maximum number of pollen grains observed on stigmas of the S-morph in cages was three, stigmas of the L-morph received up to 35 self pollen grains. This difference in autonomous self-pollination is undoubtedly caused by the small spatial separation of stigmas and anthers in the L-morph compared with the S-morph. Autonomous self-pollination in the L-morph could thus cause significant ovule discounting if cross-pollination is delayed. It is also likely that facilitated self-pollination resulting from the action of pollinators will also be greater in this morph. Data on pollen loads in distylous plants also indicate that the L-morph experiences more self-pollination than the S-morph although in this case differences in herkogamy are unlikely to be involved (Ganders, 1979; Lloyd &

Webb, 1992a; Pailler *et al.*, 2002). In *N. assoanus*, self-pollination at the same time as cross-pollination caused a significant reduction in seed set in the L-morph, whereas in the S-morph self-pollination only reduced seed-set if it occurred prior to cross-pollination. Studies of tristylous *Pontederia sagittata* have also demonstrated that the negative effects of prior self-pollination vary among style-morphs, being strongest in the L-morph (Scribailo & Barrett, 1994).

Although we were able to demonstrate the negative consequences of self-interference to maternal fitness in our controlled experiments, we found no evidence for similar effects on either maternal or paternal fitness in natural populations of *N. assoanus*. This suggests that under natural pollination conditions the two style morphs possess traits that enable them to avoid the negative consequences of self-interference. We suggest that this facility is associated with distinctive features of their floral biology, with the two morphs differing in the mechanisms that they employ to prevent self-interference.

Our field experiment indicated that the S-morph experiences little spontaneous deposition of self pollen, because of the large spatial segregation of sexual organs. Hence self-pollination in this morph is only likely to occur as a consequence of insect visitation when outcross pollen may also be delivered to stigmas. Our controlled pollination experiments indicate that in the S-morph self-pollination significantly reduces maternal fitness only if it occurs prior to the arrival of outcross pollen. This may occur infrequently under field conditions as most pollinators visiting flowers causing facilitated self-pollination are also likely to deposit outcross pollen. Hence according to this argument the principal mechanism that limits costs arising from self-interference in the S-morph is well-developed herkogamy.

In contrast, our field studies of caged flowers of *N. assoanus* indicate that the L-morph is susceptible to a high degree of autonomous self-pollination because of the close proximity of sexual organs in this morph. However, under natural conditions if pollinators are active this effect may be mitigated to some extent by the protandry exhibited by the L-morph. Our experiments on the timing of female and male function revealed that at flower opening, when upper anthers dehisce, the receptivity of stigmas of the L-morph is significantly reduced in comparison to the S-morph. This means that when viable pollen is available in the upper anthers the stigma is only weakly receptive primarily because self-pollen has difficulty adhering to stigmas. If pollinators visit flowers during this period and pollen is removed from anthers then the opportunities for the subsequent deposition of self-pollen will be reduced. It is important to note that the 1-day delay in full stigma receptivity in the L-morph corresponds to the end of dehiscence of upper anthers and to the time necessary for self-pollen to reduce seed set to levels equivalent to selfing. Hence

although flowers of *N. assoanus* are long-lived, the relatively small window of time between anther dehiscence and stigma receptivity could be functionally significant in reducing opportunities for self-interference. Future studies under natural conditions of pollen removal and deposition in relation to anther dehiscence and stigma maturity in the style morphs of *N. assoanus* would be worthwhile.

We observed no positive effect of style excision in the ability of plants of the L-morph to disperse pollen to the S-morph. The presence of a stigma close to the anthers in this morph thus does not appear to obstruct pollen removal. In the only other study that has investigated the influence of stylar interference on male function, Kohn & Barrett (1992) (using genetic markers) found no difference in male outcrossed siring success of long-styled plants of *Eichhornia paniculata* with styles and those with their styles excised. It therefore remains an open question if this proposed form of sexual interference is of functional significance in plants.

In self-compatible animal-pollinated plants marker-gene studies have demonstrated that quantitative variation in herkogamy is commonly associated with different intensities of outcrossing and in such cases well-developed herkogamy can clearly serve to reduce selfing and inbreeding depression (e.g. Holtsford & Ellstrand, 1992; Belaousoff & Shore, 1995; Affre & Thompson, 1999). However, in self-incompatible species, such as *N. assoanus*, this explanation is clearly unsatisfactory. Indeed, despite the large difference in herkogamy between the L- and S-morphs of *N. assoanus* marker-genes studies indicate that both style morphs are highly outcrossing (Baker *et al.*, 2000b). The maintenance of the herkogamy polymorphism that characterizes *Narcissus* species with stigma-height dimorphism must therefore result from selective pressures other than inbreeding avoidance. The occurrence of herkogamy in the S-morph and protandry in the L-morph suggests that these floral traits likely function as alternative strategies for limiting self-interference by reducing pollen wastage and providing greater opportunities for fitness gain through cross-pollen transfer. Although alternative mating strategies occur in various animal groups (reviewed in Shuster & Wade, 2003) the occurrence of different strategies for reducing conflict between sex functions co-existing in a polymorphic condition has not been considered before for hermaphroditic organisms.

Could self-interference contribute to the evolution of a stigma-height dimorphism?

Webb & Lloyd (1986) proposed that because self-pollination can reduce opportunities for outcrossing, selection should favour the evolution of floral traits that reduce self-interference, such as herkogamy or dichogamy, particularly in self-incompatible species. More specifically, Yeo (1975) suggested that costs associated with self-

pollination could have played a role in the origin of stigma-height dimorphism in *Narcissus*. We therefore consider next whether the levels of self-interference detected in our experimental studies may have contributed to the evolution and maintenance of stigma-height dimorphism in *Narcissus*.

Lloyd & Webb (1992a,b) proposed that stigma-height dimorphism evolves from an ancestral approach herkogamous population (i.e. monomorphic L-morph) by the invasion of a short-styled morph. Indeed, there is phylogenetic evidence that this evolutionary sequence has occurred several times in *Narcissus* (Graham & Barrett, 2004). Lloyd & Webb (1992b) modelled the necessary conditions for the establishment of this polymorphism based on cross-pollen transfer within and between morphs. Based on this model, Barrett *et al.* (1996) further explored how pollen dispersal and ovule discounting (because of self-interference) might jointly influence conditions for the establishment of a stigma-height dimorphism in *Narcissus*. They found that the conditions in which a dimorphism could be maintained are defined by two inequalities involving pollen transfer proficiencies and ovule discounting:

$$\frac{q_{ls}\left(\frac{v_s}{v_l}\right) + q_{sl}}{2} > q_{ll} \quad (1)$$

$$\frac{q_{ls}q_{sl}\left(\frac{v_l}{v_s}\right)}{2} > q_{ss} \quad (2)$$

where l and s are the two morphs, q_{ll} , q_{ss} , q_{ls} , q_{sl} are the proficiencies of pollen transfer within and among morphs and v_l and v_s are the average proportion of ovules available after ovule discounting in the L- and S-morphs, respectively. Thus, if relative ovule availability is equal (i.e. $v_l = v_s$), the average proficiency of pollen transfer between morphs should exceed the transfer proficiency among plants of the same morph for the polymorphism to be maintained. Significantly, between-morph differences in ovule discounting will modify this condition.

The results of our controlled pollinations suggest that the establishment of a stigma-height dimorphism from an ancestral approach herkogamous population could be influenced by costs associated with self-interference. If we assume that a long-styled phenotype similar in morphology to the long-styled morph of *N. assoanus*, but lacking protandry, represented the ancestral monomorphic population, then it is plausible that this phenotype would suffer from self-interference. In a population of plants with this phenotype, a novel short-styled variant (S-morph) could avoid self-interference by herkogamy, and thus have a higher proportion of ovules available for outcrossing than the ancestral L-morph. Based on our experimental results, the seed-ovule ratio in the S-morph, calculated using the results of the cross-pollination treatment (i.e. treatment 1), has a value of $v_s = 0.46$. In contrast, in the L-morph, which may suffer self-interference because of self-pollination, the average

seed-ovule ratio in the glasshouse experiment over all treatments with self-pollination (see Fig. 5), gives a value of $v_1 = 0.23$. Using these values of ovule availability and assuming relative pollen transfer proficiencies of $q_{ll} = q_{sl} = 0.5$, $q_{ls} = 0.4$, $q_{ss} = 0.3$ (see Barrett *et al.*, 1996 for discussion of the reasons why pollen transfer to the L-morph may exceed pollen transfer to the S-morph), a stable dimorphic condition is predicted. However, both morphs avoid self-interference and ovule discounting, i.e. equal ovule availability in the two morphs ($v_1 = v_s = 0.46$), based on the above pollen transfer proficiencies, inequality 2 is satisfied but inequality 1 is not. This would result in populations monomorphic for the L-morph. Therefore ovule discounting and pollen transfer may interact to influence morph ratios, with potential consequences for the evolution and maintenance of stigma-height dimorphism in *Narcissus*.

Floral hermaphroditism results in conflicts and adaptive compromises in the parental roles of plants during pollination and mating. Floral traits that reduce sexual interference between female and male function reduce gamete wastage and increase mating opportunities through more effective pollen dispersal. In most plants floral monomorphism limits prospects for the evolution of divergent adaptive strategies that reduce costs associated with sexual interference. However, in species with sexual polymorphisms, morph-specific differences in floral morphology enable the development of alternate strategies for reducing interference, such as the dichogamy and herkogamy in the L- and S-morphs respectively of *N. assoanus*. Because reproductive losses unrelated to the specific genetic costs of inbreeding depression are likely to be common in hermaphroditic plants, future studies will probably reveal other floral strategies that function to reduce sexual interference.

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