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Pollinator shifts and the loss of style polymorphism in *Narcissus papyraceus* (Amaryllidaceae)

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Abstract

Darwin proposed that the driving force for the evolution of style polymorphisms is the promotion of cross-pollination between style morphs, through accurate placement of pollen on the pollinator's body. This hypothesis has received much attention, but the effect of different pollinators in the fitness of morphs remains poorly understood. *Narcissus papyraceus* is a style dimorphic species (long -L- and short -S- styled) with isoplethic (1 : 1) and L-monomorphic populations, mainly visited by long-tongued (LT) nocturnal and short-tongued (ST) diurnal pollinators, respectively. We studied natural female fertility of morphs, and assessed the role of diurnal and nocturnal pollinators. We also quantified female fertility of the morphs in experimental populations with different morph ratio, exposed to predominately long- or short-tongued pollinators. We found that with LT pollinators, both morphs were successfully pollinated in all morph ratio conditions, suggesting that these insects could be involved in maintenance of the polymorphism, although other factors may also play a role. However, with ST pollinators, S-plants displayed less fertility than L-plants, and mating among L-plants was favoured, implying that the polymorphism is lost. These results underscore the role of pollinators on variations in style polymorphism.

Introduction

Since Darwin's time (1877), understanding the functional meaning of sex polymorphisms in plants has constituted a challenge for evolutionary biologists. He envisaged one of these polymorphisms, heterostyly, as 'adapted for reciprocal fertilization; so that the two or three forms, although all are hermaphrodites, are related to one another almost like the males and females of ordinary unisexual animals' (Darwin, 1877: 2). Ever since Darwin's statement, the cross-pollination hypothesis has attracted much attention in the study of heterostyly (e.g. Stone & Thomson, 1994; Ree, 1997; Lau & Bosque, 2003; Ornelas *et al.*, 2004; Armbruster *et al.*, 2006; Hernández & Ornelas, 2007). Furthermore, this hypothesis has broader value than originally thought as it may explain the function of other reciprocal sex polymorphisms in hermaphroditic organisms, be they plants (Barrett *et al.*, 2000; Barrett, 2002) or animals (Schilthuizen *et al.*, 2007), where mating occurs preferentially between morphs (disassortative mating).

The simplest form of heterostyly is distyly, which includes two floral forms in a population, with reciprocal placement of anthers and stigmas. Models on the evolution of heterostyly (Charlesworth & Charlesworth, 1979; Lloyd & Webb, 1992a) have proposed another sex polymorphism, style-length dimorphism (also named stigma-height dimorphism), as immediate ancestral state to distyly. Style dimorphic species present two floral forms, long- and short-styled morph (hereafter L and S respectively), with anthers remaining approximately at the same height. Lloyd & Webb (1992b) argued that the apparent rarity of style dimorphism in nature is due to its low efficiency in promoting disassortative mating. Despite the potential for style dimorphism to aid our

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understanding of the establishment and maintenance of heterostyly, there is a scarcity of studies on that polymorphism (Barrett *et al.*, 2000). According to Darwin (1877; reviewed by Lloyd & Webb, 1992a), style dimorphism and heterostyly evolves in an ecological scenario, where selective agents are pollinators transferring pollen in a precise manner, and the underlying mechanism is the promotion of cross-pollination between different morphs, by means of negative frequency-dependent selection (Pannell *et al.*, 2005).

Despite its rarity elsewhere, style dimorphism frequently occurs in the genus *Narcissus* (daffodils) (Barrett *et al.*, 1996; Arroyo, 2002; Barrett & Harder, 2005). In style dimorphic *Narcissus,* crosses within and between morphs are equally fertile, and most of species are self-incompatible (Dulberger, 1964; Barrett *et al.*, 1996; Arroyo *et al.*, 2002). Natural populations of style dimorphic *Narcissus* display a wide range of morph ratio, including isoplethic (1 : 1), L-biased and L-monomorphic populations; S-biased being rarely found (Barrett *et al.*, 1996; Arroyo, 2002), and S-monomorphic populations never reported in the wild. Research in *N. assoanus* illustrated the conditions under which the polymorphism was maintained: S-plants enhanced their female fitness when the L-morph functioned as pollen donor, particularly under L-biased conditions (Thompson *et al.*, 2003; Cesaro & Thompson, 2004). Studies on natural fruit and seed production, where the spatial arrangement of morphs was considered, also showed that disassortative mating likely occurred in S-plants (Stehlik *et al.*, 2006), whereas L-plants equally displayed assortative and disassortative mating. Despite the low reciprocity between morphs, pollination by long-tongued (LT) insects enables sufficient disassortative mating to maintain style dimorphism in all populations (Thompson, 2005).

If disassortative mating maintains the polymorphism, and were their pollination environment disappeared, a straightforward prediction would be the loss of polymorphism and fixation of a morph. This would represent a reverse test on the model of Lloyd & Webb (1992a). Indeed, L-monomorphism frequently occurs in populations of otherwise style dimorphic species (Arroyo & Dafni, 1995; Baker *et al.*, 2000a; Arroyo *et al.*, 2002; Pérez *et al.*, 2004). A pollinator-mediated selection hypothesis has been put forward to explain the loss of the S-morph: S-styles have such a concealed position within the flower tube that only LT pollinators can deliver pollen on the stigmas. L-flowers can receive pollen from either LT or short-tongued (ST) insects because L-stigmas are well exposed at the top of the floral tube. A shift towards ST pollinators would reduce pollen arrival to S-flowers and consequently female fitness of these plants (Arroyo & Dafni, 1995; Arroyo *et al.*, 2002). Available data on pollinator faunas of *N. tazetta* and *N. papyraceus* showed that dimorphic and L-monomorphic populations are visited mostly by LT and ST insects, respectively (Arroyo & Dafni, 1995; Pérez-Barrales *et al.*, 2007). However, we are still lacking experimental evidence for evaluating the effects of these different functional pollinator groups on the reproductive success of the L- and S-morph.

In our study, we aimed at examining whether a shift from LT nectarivorous to ST pollinivorous insects promotes the loss of the stylar polymorphism in populations of otherwise style dimorphic *N. papyraceus*, through concomitant changes in female fertility of L- and S-plants. We first studied natural female fertility in an isoplethic and an L-monomorphic natural population, and assessed the relative importance of diurnal (ST) and nocturnal (LT) pollinators for fruit set and seed production in both morphs. We then took advantage of the distinctive natural distribution of LT and ST pollinators of this species, and evaluated their role in female fertility in L and S-plants, under different experimental morph ratio conditions, taking into account the degree of sex organ reciprocity between morphs.

Material and methods

Study species and locations

Narcissus papyraceus (Amarillydaceae) is a self-incompatible, long-lived geophyte, commonly distributed in the Mediterranean basin, with most of its populations in the SW Iberian Peninsula and NW Africa. Within this area, populations occur on seasonally wet deep soils in open marshes, grasslands in lowlands and rarely in rocky hillsides up to 1500 m elevation. Blooming starts in late November and ends in early March, and flowering peak in coastal populations occurs in December, and in January–February in inland populations. Populations in the Strait of Gibraltar and surrounding areas are isoplethic or L-biased, whereas inland populations are L-monomorphic (Arroyo *et al.*, 2002). Vegetative reproduction occurs in dimorphic and L-monomorphic populations, without significant differences among populations (Pérez-Barrales *et al.*, 2009). Flowers have a long and narrow floral tube at the base of which nectar is produced; and a corona that produces a sweet fragrance (Dobson *et al.*, 1997). Flowers have one style, and an inferior ovary with mean ± SE of 54.8 ± 11.3 ovules ($n = 253$ flowers), and two anther levels attached to the upper side of the flower tube. The upper anther level has similar height in both morphs, but the lower anther level is located slightly lower in L-flowers (mean ± SE for six dimorphic populations, L morph: 11.6 ± 0.1 mm $n = 319$, S morph: 12.5 ± 0.1 mm $n = 229$), thus S-stigmas have some higher reciprocity with lower anthers of L-flowers (Fig. 1). The remaining perianth traits do not differ between morphs (Pérez-Barrales *et al.*, 2007), and UV reflectance patters and fragrance chemistry are identical (J. Arroyo, R. Santos, unpublished data).

Isoplethic populations are mainly visited by LT nectar-seeking pollinators (including solitary bees, butterflies, but predominantly nocturnal moths), whereas L-mono-

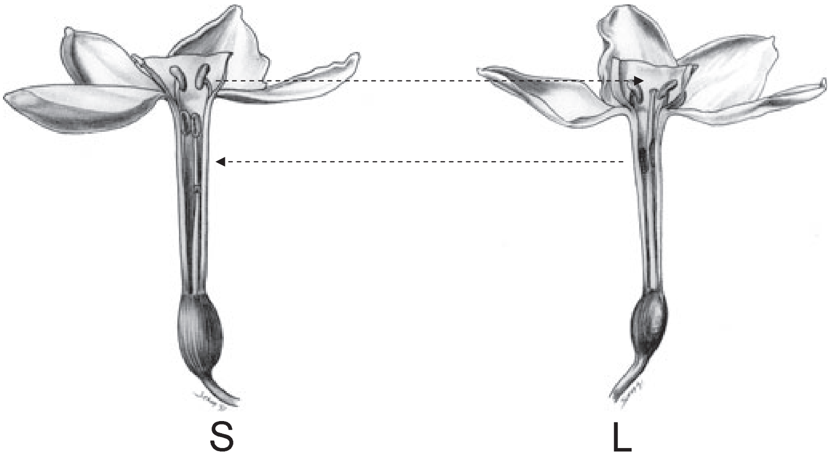

Fig. 1 Long-styled (L-morph) and short-styled (S-morph), with the relative position of stigmas and anthers of flowers of *Narcissus papyraceus*. Note that the lower anther level in the L-morph is in a lower position in the tube than in the S-morph and the reciprocity is variable between whorls, as shown by the arrows. For clarity, only two of three stamens per whorl are drawn.

morphic populations are visited almost exclusively by diurnal ST pollen-seeking pollinators (syrphid flies) and few solitary bees (*Anthophora* spp). Because the relative importance of these functional groups differs between isoplethic and L-monomorphic populations, we will respectively name the pollination environment LT and ST. The study of the pollinator fauna (Pérez-Barrales *et al.*, 2007) and experimental manipulations in the present study (see below) correspond to the same populations (isoplethic: Facinas, Cádiz prov., Spain; L-monomorphic: Aznalcázar, Seville prov., Spain) and years (2002, 2003 and 2004).

Female fertility in natural populations

We studied natural fruit set, seed production and total female fertility [product between fruit set and seed set (seed/ovule ratio)] in the isoplethic and L-monomorphic population mentioned earlier during 2 and 3 years, respectively (Table 1). We separately studied fruit set and seed production as they may inform on visitation rate and efficiency of pollinators on pollen deposition, respectively. We randomly collected fruiting stalks separated by at least two metres, estimated natural fruit set (number of fruits/number of flowers in the inflorescence) and used the first flower in the inflorescence to study seed production (number of seeds produced). We could assess the morph because this *Narcissus* species retains flower remains during fruit development.

We used a generalized linear model (GLM) to test for differences between populations and years in fruit set, seed production and total female fertility. For fruit set, we applied a GLM with binomial error distribution. For seed production, we used a GLM with Poisson error distribution. In the isoplethic population, we tested for year and morph effects, and the interaction term. For the monomorphic population, we only examined year effect. To analyse total female fertility, we used a GLM with normal distribution on angular transformed data. We compared fruit set, seed production and total female fertility between populations, using the collections of the same year (2004). We performed this last analysis in two ways: first we pooled the data of L and S plants in the isoplethic population, and then we repeated the analysis using only the data of L plants. The analyses of this study and the ones below were performed using Wald chi-square statistic for factor effects.

The role of diurnal and nocturnal pollinators in female fertility of the morphs

In the aforementioned populations, we carried out an exclusion experiment to test the effect of nocturnal and diurnal pollinators in the isoplethic (10–17 December 2003) and L-monomorphic population (18–25 January 2004). We selected 60 plants randomly distributed in each population, 30 for diurnal and 30 for nocturnal pollination. In the isoplethic population, we divided the sample into 15 L and 15 S. Plants were selected 1 day before first flower opened. To study the diurnal pollination, plants were covered with a fine mesh before sunset (1730 h approximately) and uncovered at 0800 h. Plants for nocturnal pollination were covered at 0800 h and uncovered at 1730 h. This procedure was repeated every day. We marked the number of flowers open during the experiment, transplanted plants to pots and kept them in the greenhouse of the University of Seville until the fruits had ripened, and collected them. We randomly changed the position of the plants in the greenhouse every 2 days.

We analysed fruit set, seed production and total female fertility with GLM as described previously. In the isoplethic population, we tested for treatment effect (diurnal vs. nocturnal pollinators), morph effect (L, S) and the interaction term. In the L-monomorphic population, we only tested for treatment effect. In the analysis of seed production, we included the number of flowers open during the experiment as a covariate.

Table 1. Mean ± s.e. (sample size) of fruit set, seed production and total female fertility of a natural isoplethic and L-monomorphic population of *Narcissus papyraceus,* and result of the GLM model for the temporal and spatial variation in the fruit set and seed production. The comparison between populations was done for one year (see Material and Methods for details).

	Fruit set		Seed production		Total female fertility	
	L	S	L	S	L	S
Isoplethic population						
1990	0.67 ± 0.04 (24)	0.58 ± 0.04 (27)	13.35 ± 1.95 (24)	10.22 ± 1.75 (27)	0.14 ± 0.02(24)	0.11 ± 0.02 (27)
2004	0.84 ± 0.02 (26)	0.83 ± 0.03(29)	17.79 ± 3.64 (14)	30.35 ± 5.01 (17)	0.27 ± 0.05(14)	0.28 ± 0.09 (17)
L-monomorphic population						
1988	0.7 ± 0.04 (42)		–		–	
2002	0.72 ± 0.03 (52)		28.25 ± 2.38 (52)		0.37 ± 0.03 (52)	
2004	0.71 ± 0.04 (64)		30.26 ± 2.99 (42)		0.42 ± 0.05 (42)	

	Wald-Chi square	d.f.	*P*	Wald-Chi square	d.f.	*P*	Wald-Chi square	d.f.	*P*
Isoplethic population									
Year	42.45	1	< 0.0001	70.27	1	< 0.0001	11.53	1	0.001
Morph	2.05	1	0.15	1.31	1	0.25	0.15	1	0.70
Year × Morph	0.42	1	0.52	11.98	1	0.0005	0.80	1	0.37
L-monomorphic population									
Year	0.50	2	0.78	3.20	1	0.074	1.90	1	0.17
Isoplethic vs. L-monomorphic population									
regardless the morph	19.24	1	< 0.0001	125.01	1	< 0.0001	3.42	1	0.06
L-plants only	12.78	1	0.0004	83.05	1	< 0.0001	3.32	1	0.07

In the isoplethic population, seed production for 1988 could not be estimated.

Experimental test of the effect of pollinator environment on female fertility in monomorphic and dimorphic populations

We carried out this experiment nearby the populations where natural fertility was studied and pollination environment known (LT, ST) (Pérez-Barrales *et al.*, 2007). We set the experiment at least three kilometres away from natural populations to avoid uncontrolled pollen flow into the experimental populations. We randomly established three experimental populations with contrasting morph ratios (L-monomorphic, S-monomorphic and isoplethic) with three replicates (plots) per experimental population. The distance between plots was 150 m (pollen flow is negligible at distances greater than 100 m, C. Abarca, unpublished data; as found by Barthelmess *et al.*, 2006 for similar pollinators). Plants used in the experiment were collected from a large isoplethic population. Each replicate contained 12 different plants (genets) with 3–8 blooming stalks each and set equidistant along a circumference of 2 m diameter. In isoplethic plots, L- and S-plants were located alternately, so that all plants of each morph were equidistant to both morphs. Plants were watered every 3 days. The large size of *N. papyraceus* plants, difficult to transplant and keep, limited the number of experimental populations we could set at a time ($N_{total} = 108$ plants). Thus, we first ran the experiment in the ST pollination environment (December 2001–January 2002), kept the plants in the greenhouse for 1 year and repeated the experiment in the LT pollination environment (December 2002–January 2003). For the same reason, we could not extend our design to biased morph-ratio populations.

Because we performed the experiments in the LT and ST pollination environment in two different years, we could not pool all data to compare between pollination environments. Therefore, we conducted separate analyses for LT and ST to evaluate the female fertility of morphs within the different morph ratio conditions and pollination environments. Specifically, in each pollination environment, we aimed at assessing:

1. The effect of natural morph ratio on female fertility of plants. We predict that the highest fertility should occur in the morph-ratio treatment corresponding to the natural conditions where the experiment was set (isoplethic plots in the LT pollination environment and L-monomorphic plots in the ST pollination environment).
2. Fertility of L- and S-plants in monomorphic experimental populations. We predict that fertility of L-plants should be larger than S-plants in both pollination environments because L-flowers present both stigma and upper anther level at equivalent height, whereas in S-flowers stigma height is lower than their lower anther level (see Fig. 1).

3. Fertility of L- and S-morph in isoplethic experimental populations. We do not expect differences between morphs in the LT pollination environment, as pollinators can reach both L- and S-stigmas. On the contrary, in the ST pollination environment, S-plants should be less successful than L-plants because of the concealed position of the S-stigma within the flower tube.
4. Fertility of each morph in isoplethic and monomorphic experimental populations, to test whether the presence of a morph improves the fertility of the other morph, conferring reproductive advantage to dimorphic populations. In the LT pollination environment, we expect that L-plants will be more successful in isoplethic than in L-monomorphic plots. In isoplethic plots, L-stigmas can receive pollen from the upper anther level of L- and S-donors, but also from the lower anther level of S-flowers because it is closer to L-stigmas than the lower anther level of L-plants (see Fig. 1). Accordingly, in isoplethic plots L-stigmas will receive overall more pollen (from both L- and S-flowers) than L-stigmas in L-monomorphic plots. Similarly, S-flowers will be more successful in isoplethic than S-monomorphic plots due to a more reciprocal position of the lower anther level of L- than S-flowers (see Fig. 1). In the ST pollination environment, hoverflies can only reach the upper anther whorl in both morphs; therefore, we do not expect differences in fertility of L-flowers in isoplethic and monomorphic plots. Fertility in S-plants in both isoplethic and S-monomorphic plots should be low and differences nonsignificant because of the inability of ST pollinators to transfer pollen to S-stigmas.

We used the first five flowers within the inflorescence to estimate fruit set, seed production and total female fertility. We used generalized estimating equation option within GLM module of SPSS v. 15 (SPSS, 2006) to study fruit set (binomial error distribution) and seed production (Poisson error distribution), setting plant (genet) as repeated measure because the response variable is not independent within plants. To analyse total female fertility, we used a GLM with normal distribution on angular transformed data. In the analyses for predictions #1 and #4, plots were nested within the experimental populations (morph ratio treatment); for prediction #2 plots were nested within morph; and for prediction #3 we included the interaction term morph × plot. We also included flower position within inflorescence in analyses for fruit set and seed production, to indicate the order in which flowers and fruits are produced (1–5). We adjusted for type 1 error using the Bonferroni correction (Sokal & Rohlf, 1995) to account for multiple comparisons within the same analysis. There were no significant differences in either flower number per plant in the plots (ANOVA: LT, mean ± SE, 26.01 ± 2.04 flowers, $F_{7,85} = 1.33$, $P = 0.25$; ST, 25.27 ± 2.28 flowers, $F_{7,83} = 0.84$, $P = 0.56$), or in number of flowers per morph in the isoplethic plots (ANOVA: LT, $F_{1,19} = 0.40$, $P = 0.54$; ST, $F_{1,21} = 2.39$, $P = 0.14$), so these variables were not included in the analyses.

Results

Female fertility in natural populations

In the isoplethic population, we did not find significant differences between morphs in fruit set, seed production or total female fertility, but year effect was significant as all fertility measures differed between years. The interaction term (morph × year) was significant only for seed production (Table 1) indicating that differences in seed production across years were variable between morphs. In the L-monomorphic population only seed production marginally differed between years. The comparison between populations showed that fruit set was higher in the isoplethic than the L-monomorphic population, but the difference was opposite for seed production (Table 1). Differences between populations in total female fertility were marginally significant (Table 1), with the L-monomorphic population displaying larger fertility values than the isoplethic population.

Effect of diurnal and nocturnal pollinators on female fertility of the morphs

The pollinator exclusion experiment in the isoplethic population showed no difference in fruit set between nocturnal or diurnal visits, but seed production was significantly higher after nocturnal than diurnal visits (Table 2, Fig. 2). L-plants had higher fruit set and seed production than S-plants, both after diurnal and nocturnal pollination. The interaction term (pollination treatment × morph) was only significant for seed production (Table 2), with seed production of S-plants proportionally higher after nocturnal than diurnal pollination (Fig. 2). For total female fertility, we found the same pattern as in fruit set, except that morph effect was only partially significant (Table 2). In the L-monomorphic population, fruit set, seed production and total female fertility were significantly lower after nocturnal than diurnal visitors (Table 2, Fig. 2). The effect of number of flowers open during the experiment was only significant in the isoplethic population.

Female fertility, pollinator environment and experimental morph ratio

We predicted that isoplethic plots would have higher fertility than monomorphic plots in the LT pollination environment; whereas in the ST pollination environment L-monomorphic plots would perform better than isoplethic or S-monomorphic plots. In both LT and ST pollination environments, there were significant differences between morph-ratio treatments in all fertility estimates in the predicted directions (Tables 3 and 4),

Table 2 Results of the generalized linear model model to assess the influence of diurnal and nocturnal pollinators (by means of an exclusion experiment) on the fruit set, seed production and total female fertility in an isoplethic and an L-monomorphic natural population.

	Fruit set			Seed production			Total female fertility		
	Wald-Chi square	d.f.	*P*	Wald-Chi square	d.f.	*P*	Wald-Chi square	d.f.	*P*
Isoplethic population									
Treatment	1.69	1	0.19	60.06	1	< 0.0001	1.97	1	0.16
Morph	9.64	1	0.005	45.86	1	< 0.0001	2.79	1	0.09
Treatment × Morph	0.70	1	0.15	13.31	1	< 0.001	0.11	1	0.73
# flowers open	–	–	–	8.88	1	0.003			
L-monomorphic population									
Treatment	11.13	1	< 0.0001	87.21	1	< 0.0001	9.66	1	0.002
# flowers open	–	–	–	1.57	1	0.21			

particularly in the ST pollination environment, where L-monomorphic plots displayed the highest female fertility by far (Fig. 3).

In the monomorphic treatment, our prediction that L-monomorphic plots would be more successful than S-monomorphic plots in both pollination environments was not fully confirmed (Tables 3 and 4). Only in the ST pollination environment, L-monomorphic plots had larger fertility than S-monomorphic plots (Fig. 3); whereas in the LT pollination environment, fertility of S-monomorphic plots was larger than L-monomorphic plots (except for seed production) (Fig. 3).

For the isoplethic plots, we expected differences between morphs only in the ST pollination environment, where L-plants were expected to perform better than S-plants. The results supported this prediction for fruit set and seed production, but not for total female fertility in the ST pollination environment (Table 4, Fig. 3). Differences in fertility between morphs were not significant in the LT pollination environment, as predicted (Table 3, Fig. 3).

Our last test aimed to assess what pollination environment prompts mating between L- and S-morphs (which can only occur in isoplethic plots) rather than within them. In the LT pollination environment, we predicted that L-plants in isoplethic plots would show higher fertility than L-plants in L-monomorphic plots, which was confirmed. We expected S-plants to have higher fertility in isoplethic than S-monomorphic plots, but this hypothesis was not supported as fertility of S-plants did not differ between isoplethic and S-monomorphic plots (Table 3, Fig. 3). In the ST pollination environment, we predicted that fertility of L-plants would be similar in isoplethic and L-monomorphic plots, but we found that L-plants were more successful in L-monomorphic plots than isoplethic plots (except for seed production). For S-plants, we predicted that fertility would be very low in isoplethic and S-monomorphic plots. We partially confirmed our expectations: fertility of S-flowers was very low, but significantly higher in isoplethic than in S-monomorphic plots (Table 4, Fig. 3).

Flower position had a strong significant effect on fruit seed and seed production in all analyses in both LT and ST pollination environment, and variation among plots, or its interaction, was significant only in the LT pollination environment, except for total female fertility (Tables 3 and 4).

Discussion

Natural morph fertility and pollinator types

Female fertility in the isoplethic population differed across years, but there were no differences between morphs, although some between year differences affected morphs differently (significant interaction term). These results agree with those found in other style dimorphic species (Baker *et al.*, 2000b), where authors surveyed several dimorphic populations in 2 years, and could not detect any fitness advantage associated to a morph. In contrast, there were not differences between years in fertility in the L-monomorphic population. The comparisons between populations showed that fruit set was significantly higher in the isoplethic population, but seed production was higher in the L-monomorphic population. Overall, these results suggest that pollination environment is probably less variable in the L-monomorphic than in the isoplethic population. The different pollinator assemblages may explain in part these differences. The L-monomorphic population is mostly pollinated by hoverflies (Pérez-Barrales *et al.*, 2007) which have some thermoregulatory capacities (Morgan & Heinrich, 1987). This could confer them some independence to weather variations. Lepidoptera, the main pollinator group in the isoplethic population, are very sensitive to weather variation (Yela & Holyoak, 1997), and their abundance often changes across years (Stefanescu *et al.*, 2003). However, we lack data on other dimorphic and L-monomorphic populations and longer temporal sampling, thus our findings should be taken with caution. Differences in fruit set and seed production patterns may inform about the efficiency of pollinators. Hoverfly pollination proved highly efficient for all

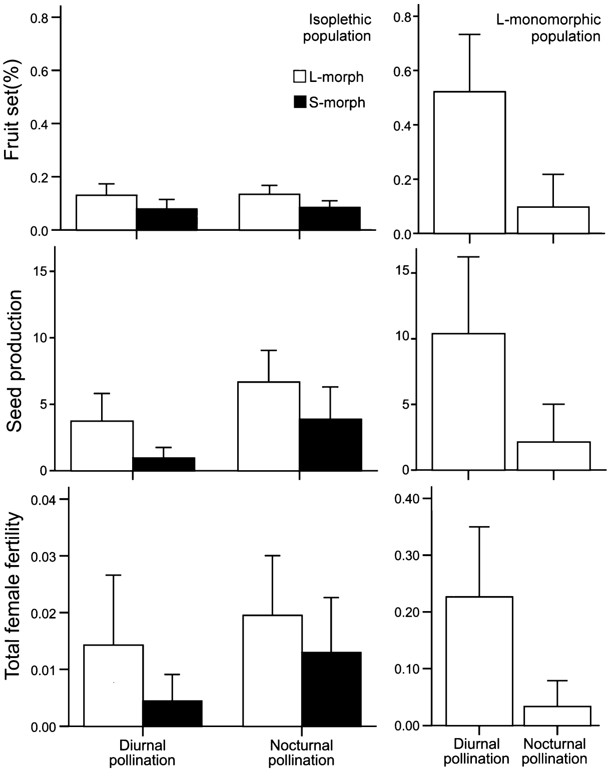

Fig. 2 Mean and 95% CI of fruit set, seed production and total female fertility of the L (open bars) and S morph (filled bars) in a natural isoplethic population (L:S) and a natural L-monomorphic population (L) of *Narcissus papyraceus*, under two experimental treatments: diurnal and nocturnal pollination. The floral visitors in the isoplethic population mainly included long-tongued insects; whereas in the L-monomorphic, population were diurnal short-tongued insects (see Material and methods).

fertility measurements in L-monomorphic populations. In isoplethic populations, fertility values were more variable, possibly as a result of the diverse array of pollinators, which includes Diptera, Hymenoptera and Lepidoptera (Pérez-Barrales *et al.*, 2007), all of them differing in their efficiency in pollen transfer to stigmas (Jennersten, 1984; Herrera, 1987).

The exclusion experiment demonstrated the distinct contribution of diurnal and nocturnal pollinators to female fitness of each morph, as previously suggested (Pérez-Barrales *et al.*, 2007). In the isoplethic population, L-plants produced more fruits than S-plants, independent of exclusion treatment. L-stigmas are more exposed, and they can receive pollen from either LT or ST visitors, irrespective of their efficiency. However, nocturnal visitors increased the seed production of both morphs but proportionally more on S-plants. All nocturnal visitors (moths) are LT insects, which are able to reach S-stigmas. The few LT diurnal visitors (mostly solitary bees) determined very low seed production in S-flowers. This greater fertility after nocturnal pollinators is a probable consequence of the high efficiency (in terms of both quality and quantity of pollen deposited on stigmas) of Lepidoptera as pollinators (for instance as a result of longer flight distance; Herrera, 1987; Lind, 1994). In the L-monomorphic population, we found much higher fruit set and seed production after diurnal pollination, due to the intense activity of ST hoverflies.

In species with nocturnal a pollination syndrome, contribution to plant female fitness by nocturnal insects is usually greater than diurnal insects (Young, 2002; Barthelmess *et al.*, 2006, but see Wolff *et al.*, 2003 and Giménez-Benavides *et al.*, 2007). The long and narrow flower tube of *N. papyraceus* flowers facilitates close fit with the long-proboscis of nocturnal visitors. This close fitting particularly benefits S-flowers, as it might promote high rates of pollen deposition (Medan, 1991, 2003; Nishihiro *et al.*, 2000; Massinga *et al.*, 2005). The importance of different pollinator assemblages (all diurnal) in promoting differential pollination success in both morphs of distylous species has been suggested (Ornduff, 1975; Beach & Bawa, 1980). To our knowledge, this is the first study reporting a different role of nocturnal (LT) and diurnal (mostly ST) pollinators in female fitness of a style polymorphic species. Absence of nocturnal visitors in inland L-monomorphic populations is probably due to low temperatures during most of the mid-winter blooming season of *N. papyraceus* (frost risk: 2–20 days; mean monthly minimum temperature Dec: 7.1, Jan: 5.7, Feb: 6.9 °C) than in the coastal region, where isoplethic populations spread (frost risk: 0 days; mean monthly minimum temperature Dec: 12.7, Jan: 11.4, Feb: 11.5 °C; source: http://www.aemet.es).

Effect of experimental morph ratio in different pollination environments

The experimental setting allowed making predictions based on flower morphology (reciprocity between equivalent sex organs of morphs; Fig. 1), morph ratio and pollinator tongue length (long or short). In short, we predicted that in presence of LT pollinators, both morphs would display similar fertility, whereas with ST pollinators, L-plants would be more successful than S-plants. In general, our predictions were supported.

Long-tongued pollination environment

Isoplethic plots displayed higher fertility than monomorphic plots, and we could not detect any fitness advantage associated to any morph in experimental isoplethic plots. These two results support that LT pollinators favour this morph-ratio, which is the condition in natural populations in that environment. We also confirmed the predicted higher fertility of L-plants in isoplethic than in L-monomorphic plots for seed production and total female fertility, probably because of an improved pollen transfer to L-plants from S-plants (see prediction # 4 in

Table 3 Results of the generalized estimated equations for the different models to test for the effects of long-tongued pollination environment (see text for details) on the fruit set and seed production, and the generalized linear model on total female fertility of the L and S morph of *Narcissus papyraceus* in experimental populations with different morph ratio (L-momomorphic; S-monomorphic; isoplethic). Significant *P* values after the Bonferroni adjustment are in bold.

		Long-tongued pollination environment								
		Fruit set			Seed Production			Total female fertility		
Model (planned comparisons)	Predictors	Wald-Chi square	d.f.	*P*	Wald-Chi square	d.f.	*P*	Wald-Chi square	d.f.	*P*
Overall effect of treatment	Morph ratio	10.60	2	**< 0.005**	10.59	2	**< 0.005**	25.65	2	**< 0.0001**
	Plot (morph ratio)	37.25	5	**< 0.0001**	15.93	5	**< 0.007**	12.61	5	0.03
	Flower position	122.59	4	**< 0.0001**	130.37	4	**< 0.0001**	–	–	–
Monomorphic plots (S and L)	Morph of the plot	9.67	1	**< 0.002**	4.05	1	0.044	7.32	1	**0.01**
	Plot (morph)	34.57	4	**< 0.0001**	15.54	4	**< 0.004**	15.09	4	**0.005**
	Flower position	78.08	4	**< 0.0001**	104.91	4	**< 0.0001**	–	–	–
Isoplethic plots (S:L)	Morph of the plant	0.94	1	0.33	1.10	1	0.274	0.57	1	0.45
	Plot	0.06	1	0.83	0.13	1	0.717	0.09	1	0.76
	Morph of the plant × Plot	0.34	1	0.56	0.28	1	0.595	0.002	1	0.96
	Flower position	24.04	4	**< 0.0001**	29.11	4	**< 0.0001**	–	–	–
L plants (monomorphic vs. isoplethic plots)	Morph ratio	0.26	1	0.61	9.35	1	**< 0.002**	18.81	1	**0.001**
	Plot (morph ratio)	34.95	3	**< 0.0001**	15.70	3	**< 0.001**	14.91	3	**0.002**
	Flower position	71.57	4	**< 0.0001**	77.91	4	**< 0.0001**	–	–	–
S plants (monomorphic vs. isoplethic plots)	Morph ratio	0.22	1	0.64	0.15	1	0.696	0.47	1	0.49
	Plot (morph ratio)	0.95	3	0.81	0.77	3	0.857	0.25	3	0.97
	Flower position	32.34	4	**< 0.0001**	56.90	4	**< 0.0001**	–	–	–

Table 4 Results of the generalized estimated equations for the different models to test for the effects of short-tongued pollination environment (see text for details) on the fruit set and seed production, and the generalized linear model on total female fertility of the L and S morph of *Narcissus papyraceus* in experimental populations with different morph ratio (L-momomorphic; S-monomorphic; isoplethic experimental plots). Significant *P* values after the Bonferroni adjustment are in bold.

		Short-tongued pollination environment								
		Fruit set			Seed Production			Total female fertility		
Model (planned comparisons)	Predictors	Wald-Chi square	d.f.	*P*	Wald-Chi square	d.f.	*P*	Wald-Chi square	d.f.	*P*
Overall effect of treatment	Morph ratio	74.87	2	**< 0.0001**	49.546	2	**< 0.0001**	109.84	2	**< 0.0001**
	Plot (morph ratio)	7.75	5	0.17	8.807	5	0.12	4.74	5	0.45
	Flower position	125.02	4	**< 0.0001**	95.773	4	**< 0.0001**	–	–	–
Monomorphic plots (S and L)	Morph of the plot	69.37	1	**< 0.0001**	43.223	1	**< 0.0001**	54.29	1	**< 0.0001**
	Plot (morph)	7.57	4	0.12	8.532	4	0.07	4.90	4	0.3
	Flower position	72.69	4	**< 0.001**	62.053	4	**< 0.0001**	–	–	–
Isoplethic plots (S:L)	Morph of the plant	26423.62	1	**< 0.0001**	54.584	1	**< 0.001**	0.08	1	0.78
	Plot	0.44	1	0.51	0.015	1	0.9	0.03	1	0.87
	Morph of the plant × Plot	0.24	1	0.63	1.089	1	0.3	0.01	1	0.94
	Flower position	60.70	4	**< 0.0001**	88.003	4	**< 0.0001**	–	–	–
L plants (monomorphic vs. isoplethic plots)	Morph ratio	11.63	1	**< 0.0001**	3.604	1	0.06	9.22	1	**0.002**
	Plot (morph ratio)	2.01	3	0.57	0.404	3	0.97	0.41	3	0.94
	Flower position	63.45	4	**< 0.001**	60.720	4	**< 0.0001**	–	–	–
S plants (monomorphic vs. isoplethic plots)	Morph ratio	34.72	1	**< 0.0001**	18.731	1	**< 0.0001**	17.12	1	**< 0.0001**
	Plot (morph ratio)	5.69	1	0.13	9.171	1	0.03	4.52	3	0.21
	Flower position	72.69	4	**< 0.0001**	124.445	4	**< 0.0001**	–	–	–

Material and methods and Fig. 1). It was unexpected to find high and similar fertility of S-flowers in S-monomorphic and isoplethic plots, suggesting that they equally received pollen from L- and S-plants. These findings contrast with previous results in the style dimorphic *N. assoanus*, where mating was random in the L-morph and mostly disassortative in the S-morph (Thompson *et al.*, 2003; Cesaro & Thompson, 2004; Stehlik *et al.*,

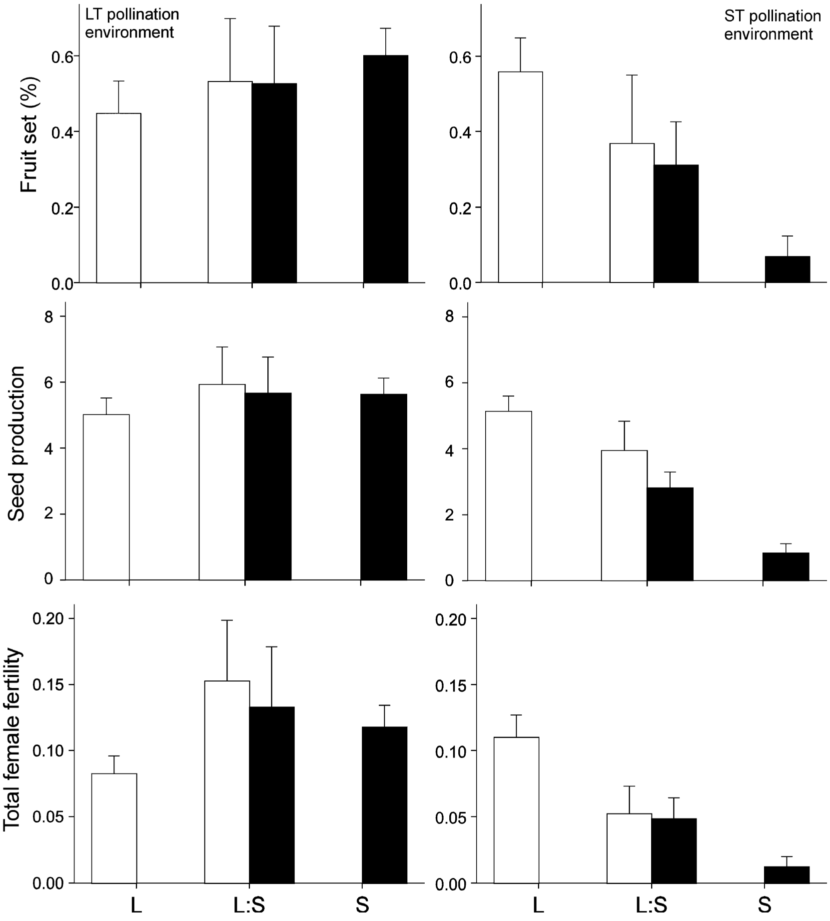

Fig. 3 Mean and 95% CI fruit set, seed production and total female fertility of the L (open bars) and S morph (filled bars) of *Narcissus papyraceus* under different pollination environments and experimental morph ratio. The three treatments, L, (L-monomorphic), L:S (isoplethic) and S (S-monomorphic) were set in two areas within the natural distribution of the species with two contrasted pollination environments dominated by long-tongued (LT) and short-tongued (ST), respectively.

2006). These authors argued that disassortative mating in S-plants was a consequence of the relatively high reciprocity between the S-stigma and the lower anther level of the L-morph, and a well-developed reverse herkogamy, which prevented assortative mating. Herkogamy remarkably varies among *Narcissus* species (Pérez *et al.*, 2004), and it is smaller in S-flowers of *N. papyraceus* than *N. assoanus* (mean ± SE for six dimorphic populations in *N. papyraceus*: 2.6 ± 0.2 mm; *N. assoanus* 5.9 ± 0.2 mm, data from Cesaro & Thompson, 2004), meaning that lower anther level of S-flowers is closer to S-stigmas in *N. papyraceus* than *N. assoanus*. These morphological differences might influence mating patterns, and under adequate LT pollinators, S-plants of *N. papyraceus* could mate with either L- or S-plants.

Short-tongued pollination environment

Fertility of L-plants was higher in monomorphic than isoplethic plots, which suggests that mating among L-plants (assortative) was favoured. These results support the fit of L-monomorphic population to the ST pollinator environment, where natural populations are L-monomorphic (Arroyo *et al.*, 2002). Besides, it was surprising that L-plants in isoplethic plots showed lower fertility than in L-monomorphic plots, as L- and S-flowers have their upper-stamen whorl at similar height and are equally accessible to ST hoverflies (*Eristalis* spp) that feed in these anthers. A careful examination of the source natural population showed that the upper-anther height in L-flowers is closer to the L-stigma than the upper anther level of S-flowers (L-stigma: 17.0 ± 0.3 mm; L upper anther level: 15.9 ± 0.3 mm; S upper anther level: 15.6 ± 0.3 mm). This difference could improve reciprocity among L-flowers than between L- and S-flowers. Small differences in the anther level and fine adjustment between pollinators and position of flower sex organs influence mating patterns in plant populations (Conner *et al.*, 1996; Cresswell, 2000). Fertility of S-plants was lower than L-plants, both in monomorphic and isoplethic plots, providing strong support to the hypothesis that pollination service for S-flowers is reduced in a ST pollination environment (Arroyo & Dafni, 1995; Arroyo *et al.*, 2002). However, the presence of L-plants improved the fertility of S-plants compared to those in S-monomorphic plots (Fig. 3), probably because of a somehow higher reciprocity between morphs (see Fig. 1) that, in presence of some Anthophorid bees, could enhance the pollen transfer from L- to S-flowers. These bees are relatively scarce in comparison with hoverflies (Pérez-Barrales *et al.*, 2007), and probably are not sufficient as to guarantee high fertility of S-plants, which never reached the fertility values of L-plants.

Overall, our results showed that LT insects equally pollinated both morphs, but these insects also determined high mating levels among S-plants. Therefore, under the adequate pollination environment S-monomorphic populations of *N. papyraceus* could exist (see Medan, 1991, 2003 for similar cases); but so far, these have never been found in nature. This is likely due to the inheritance of the style morph: a single Mendelian locus, with S-morph determined by a dominant allele in heterozygosis (Ss) and L-morph by recessive allele (ss) (Dulberger, 1964). In absence of fitness advantage, populations founded by S-plants would sire both morphs, making generation of an S-monomorphic population virtually impossible. On the contrary, the establishment of S-flowers in a ST pollination environment (either by migration or mutation) would be seriously hindered because of a very low fertility of S-plants compared to L-plants, an enhanced assortative mating among L-flowers (Fig. 3), and the homozygosity of L-flowers for the style length locus (Dulberger, 1964).

Concluding remarks

The Darwinian cross-pollination hypothesis for the evolution of stylar polymorphisms (Lloyd & Webb, 1992b) predicts that dimorphism should be maintained through high levels of mating between morphs, serviced by appropriate pollinators. If these are lost or replaced by less efficient pollinators, polymorphism should be lost. Correlative studies have suggested that the loss of the style polymorphism driven by pollinators occurs across populations (Arroyo & Dafni, 1995) and lineages (Pérez-Barrales *et al.*, 2006). Here, we provide functional evidence at population level: shifts towards less efficient pollinators in transferring pollen between morphs can reduce female fitness of S-plants, as it occurs in natural monomorphic populations pollinated by ST hoverflies. Under these conditions, spreading of the S-morph into L-monomorphic populations should be prevented. Our data are less clear about the effect of LT pollinators on the maintenance of style dimorphism; although they seem to play a role, as S-plants pollinated by them always showed high fertility. However, morph inheritance is also critical. Discriminating among these factors would require fine estimates of the male fitness component of both morphs, determining levels of assortative and disassortative mating at the plant level (Hodgins & Barrett, 2008; Rosas & Domínguez, 2009), and modelling the effect of these mating patterns on the morph ratio of subsequent generations. Our results of pollinator effects on style morph fertility are complementary to those by Pérez-Barrales *et al.* (2007), which showed that these different pollinators also select for different perianth trait combinations in dimorphic and monomorphic populations, thus they illustrate a more general picture on the effects of pollinator shifts on moulding flower phenotypes in populations.

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