Floral morphology and reproductive success in herkogamous Narcissus cyclamineus (Amaryllidaceae)

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Abstract We studied the variation on floral traits of Narcissus cyclamineus, a species endemic to the northwestern Iberian Peninsula. We analyzed the effect of different breeding systems and the degree of herkogamy and stigmatic exertion on female reproductive success. Results showed that, while variation on floral structures is relatively low (i.e. less than 25% for all sterile structures), CVs for herkogamy and stigmatic exertion are much higher. We also found a considerable variation among populations in the relative position of stigmas and anthers and a significant effect of breeding system (facilitated autogamy, spontaneous autogamy or xenogamy) on fruit set and seed production. The number of seeds per fruit bears no relation with corona size, degree of stigmatic exertion or herkogamy. However, herkogamy showed a marginally significant effect on mean seed weight per fruit. The results highlight the need to analyze the influence of continuous variations in the position of floral organs on the reproductive success of the monomorphic species of the genus Narcissus.

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Introduction

The grouping of male and female gametes into specialised reproductive structures, together with the possibility of self-fertilisation, represent two essential characteristics of hermaphrodite plants which have undoubtedly contributed to the extraordinary diversity of sexual systems (Barrett 2003). Traditionally, many of the variations in reproductive systems have been interpreted as mechanisms that promote outcrossing (Barrett and Harder 1996). For example, the spatial and temporal segregation of sex organs, within and between flowers, may act as an 'anti-selfing mechanism', preventing the adverse effects of selfing and inbreeding depression (Lloyd and Webb 1986). However, many species exhibiting this trait are self-incompatible and therefore, already protected from these negative effects. In such cases, the segregation of sex organs may act by reducing interference between male and female functions and enhancing pollen export efficiency (Lloyd and Webb 1986; Webb and Lloyd 1986; Harder and Barrett 1996; Barrett 2002, 2003; Medrano et al. 2005).

One of these mechanisms, shared by animal-pollinated plants, is the spatial separation of pollen presentation and pollen receipt, also known as herkogamy. Several different forms of herkogamy are known to exist, but the most common, by far, is approach herkogamy, a strategy by which stigmas are located above the level of the anthers, causing pollinators to first contact the stigma as they enter the flowers (Webb and Lloyd 1986; Barrett 2003). Avoidance of selfing has also been cited as a driving force underlying the

evolution of herkogamy. In fact, while far from being a general pattern (Medrano et al. 2005), the distance separating stigmas and anthers was found to influence maternal outcrossing rates in several self-compatible species (see Belaoussoff and Shore 1995; Karron et al. 1997; Brunet and Eckert 1998). The degree of herkogamy may also be considered a trade-off between the reduction of sexual interference and the increase in pollen transfer precision (Barrett 2002). By decreasing sexual interference, the flower may reduce the amount of pollen loss on its own stigma (pollen discount), resulting in a reduction of the mean investment per dispersed pollen grain (Barrett 2002 and references therein). If herkogamous flowers show increased outcrossing rates, we would expect a higher female reproductive success related to herkogamy. On the contrary, an increase in the reproductive success of the male function would occur if the reduction of sexual interference is what drives the evolution of herkogamy.

The genus Narcissus encompasses 65 species of perennial geophytes distributed throughout the western Mediterranean region (Arroyo 2002), featuring species that are monomorphic, dimorphic and trimorphic for style length. Furthermore, the genus includes monomorphic taxa, both self-compatible (e.g. N. longispathus) and self-incompatible (e.g. N. bulbocodium), together with others with no evidence of any linkage between style polymorphism and the incompatibility reaction (N. assoanus) (Herrera 1995; Barrett et al. 1996; Barrett et al. 2004; Graham and Barrett 2004; Barrett and Hodgins 2006). This wide diversity of sexual systems has made the genus Narcissus an excellent model for understanding the evolution of floral polymorphisms, despite the fact that information on the natural history of some taxa is still lacking.

Narcissus cyclamineus is a species endemic to the northwestern part of the Iberian Peninsula and is included in the Pseudonarcissus section, one of the ten recognized for this genus. Although the species has been described as self-incompatible (Bateman 1954), preliminary data have revealed high levels of fruit set across the populations, despite very low rates of pollinator visits (Larrinaga et al. unpublished data), suggesting that this species might show a certain degree of self-compatibility. As this species exhibits a limited floral display (one flower per plant), its self-compatibility makes it a suitable model for the analysis of the functional role of herkogamy.

In this work, we analyze the morphological variation in the floral traits of N. cyclamineus, focusing particularly on the female function. The following specific questions have been addressed: (1) What is the effect of the different breeding systems (namely, facilitated autogamy, spontaneous autogamy, and xenogamy) on fruit set and seed production? and (2) What is the effect of the degree of herkogamy and stigmatic exertion on female reproductive success?

Methods

Study species and area

Narcissus cyclamineus DC. (Amaryllidaceae) is a 20–30 cm bulbous geophyte that appears along the banks of watercourses and damp meadows in the Atlantic catchment areas of the northern Iberian Peninsula. Its scape develops a single, pendulous yellow flower with a 15–25 mm spathe. The corona is cylindrically tubular enclosing six equal sized stamens and a discoidal stigma with a variable degree of herkogamy. Flowering takes place from February to March, and fruits ripen from June to July. Fruit set levels are high in all populations (range 70–90%; Larrinaga et al., unpublished data). The reproductive and vegetative structures of the plant are subject to heavy predation by slugs and rodents. A recent prospecting study of potential distribution areas enabled us to locate 46 populations, ranging in number from 5 to 10,000 flowering individuals (Larrinaga et al. unpublished data).

The study was carried out on four populations in the province of A Coruña (Spain): Grixoa (Santiago de Compostela, number of reproductive individuals within the population = 1,646), Santa Comba (n = 562), Castro (Zas, n = 9,880) and Trasufre (Muxia, n = 450). All the populations are located in separate, geographically isolated river basins.

Floral morphology

For each population 41–45 flowers were collected and immediately placed in plastic bags and taken to the laboratory to prevent wilting. Once there, by means of a digital calliper, the following measurements were recorded (Fig. 1): corona length (L_{cor}), corona diameter (D_{cor}), corona aperture (A_{cor}), stamen length (L_{sta}), anther length (L_{ant}), filament length (L_{fil}), and style length (L_{sty}) (Fig. 1). The degree of stigmatic exertion was calculated for each individual flower as $E = L_{sty} - L_{cor}$, and herkogamy was computed as $H = L_{sty} - L_{sta}$. Therefore, positive values of stigmatic exertion indicate exerted stigmas, and positive herkogamy values point to the existence of approach herkogamy. We did not find any flowers with reverse herkogamy, as the negative values of herkogamy in our samples corresponded to flowers whose stigmas are at the same level as the anthers, but never lower than their proximal end.

Breeding systems

To analyze the effects of different breeding systems on reproductive success 90 plants distributed throughout the populations of Castro and Trasufre (45 each) were bagged with tulle. Once anthesis took place, we pollinated 15 plants
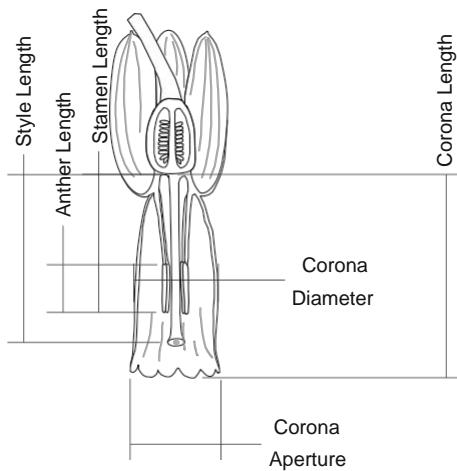
Fig. 1 Schematic drawing of Narcissus cyclamineus flower and the measures collected for the present study. Derived measures were calculated as follows: filament calculated as stamen length—anther length, exertion as style length—corona length, and herkogamy as style length—stamen length (see text for details)

with their own pollen (facilitated autogamy) in each population, and 15 with pollen from other local individuals (after removing the stamens with pincers; xenogamy), leaving another 15 individuals unmanipulated to be used as experimental controls (spontaneous autogamy). Each flower subjected to the xenogamy treatment received a mixture of pollen from ten different donors which were haphazardly selected from the whole population, avoiding the immediate vicinity of the focal flower. When fruits ripened, bags were removed and seeds were individually counted and weighed (with a 10^{-1} mg precision balance). Fruit ripening was assessed by their yellowish color, which occurs one or two days before fruit opening. N. cyclamineus often produces non-viable seeds, which despite being blackish in color like healthy mature seeds, are empty and easy to recognize. Although these aborted seeds were counted and weighed, they were omitted from the analyses, except in cases where "aborted seed number" was included as a covariate. Otherwise, the number of seeds always refers to mature seeds.

Stigmatic exertion and approach herkogamy

In order to study the effect of approach herkogamy on female reproductive success, we selected 30 individuals in the Castro population, spanning a substantial gradient of separation between stamens and stigma. To prevent involuntary pollen transfer, small, disposable paper rulers were used to measure the distance between the stigma and the corona rim, as well as the distance between the latter and the closest end of the anthers with a precision of 1 mm. The plants were left in natural pollination conditions and once fruit setting took place, individuals were bagged to avoid predation. Mature fruits were then collected and the seeds were counted and

weighed. To check for the presence of ontogenic changes in herkogamy, we collected 25 plants presenting flower buds. Once in the lab, they were planted in individual pots and their herkogamy was measured every day from the start of the anthesis to the end of the flowering period.

Data analysis

Variation in floral morphology traits among the four populations was assessed by means of univariate one way ANOVAs. Bivariate correlation anaylses between the flower traits measured were also performed for the whole sample. As the number of ANOVAs and correlation analyses performed was high, Bonferroni's sequential correction was applied (Rice 1989).

To prevent the breeding system experiment from failing, owing to any adverse circumstances that could affect a particular sampling site, the trial was conducted on two populations, although this study did not aim to evaluate any Population effect. Thus, the Population effect was included as a fixed factor in all the analyses in order to control the differences that might arise between these two populations.

The effect of pollination treatment on fruit set and its variation among populations was analyzed by means of a Generalized Linear Model, with data adjusted to a binomial distribution and 'logit' as the link function, including pollination treatment and population as fixed factors. We reduced the model by eliminating the effects that presented a $P > 0.25$, following the recommendations of Winner et al. (1991) and Underwood (1997). Moreover, in all cases the model selected in this way presented the lowest AIC. All P values were estimated by means of the type III likelihood ratio test.

The effect of pollination treatment on seed production and its variation among populations was analyzed by means of an ANOVA. The response variable (number of seeds) was square root transformed. Population and pollination treatment were considered fixed factors. Plants whose fruit was already open when collected were discarded, since the small seed size and the bagging system could not guarantee the collection of every seed. Flowers that did not set fruit were not considered in this analysis. Two a priori contrasts were also performed, one testing facilitated versus spontaneous autogamy treatment, and the other testing facilitated autogamy versus xenogamy treatment.

The effect of the pollination treatment on seed weight was analyzed by a General Linear Model with slope heterogeneity. Population and pollination treatment factors were considered fixed and the square root of the number of ripe seeds was included as a covariate. Fruits already open when collected and those having no ripe seeds (due to the inclusion of ripe seed number as a square root transformed covariate) were discarded. As in previous analyses, the full

model was simplified by progressively eliminating the effects presenting the highest P values, provided that they were greater than 0.25 (Winner et al. 1991; Underwood 1997). The final model showed the lowest AIC value of all the models tested. The fact that there is an interaction between the covariate and the factors detracts from the latter's significance (Huitema 1980). For this reason we resorted to a Johnson–Neyman analysis (Huitema 1980) to calculate the regions of significance.

Finally, we analyzed the influence of approach herkogamy and the degree of stigmatic exertion on the number and weight of the seeds of the unmanipulated flowers in our herkogamy–exertion experiment (see above), through a multiple regression where both seed number variables (mature and aborted) were square root transformed. The effect of corona length was included to monitor the possible influence of individual plant size. Variable selection was performed as in previous analyses (see above).

The daily evolution of herkogamy was analyzed by means of a Generalized Linear Mixed Model, with a repeated measures design, where time of measurement was the within-subject factor.

All analyses were performed using Statistica 6.0 and SPSS 11.5, and 14 software. For the Johnson–Neyman analyses, we modified the routines described by O'Connor (1998) to take into account the number of comparisons carried out after Huitema (1980).

Results

Floral morphology

Narcissus cyclamineus is a monomorphic species which presents considerable variation in the relative position of stigmas and anthers (Fig. 2). Descriptive statistics for all flowers show that, overall, most variables exhibited a CV of around 10–20%, except stigmatic exertion and herkogamy level whose CV values were very high. Variation among populations was significant in corona diameter and aperture, as well as in sex organ-related traits (stamen and style length, and the degree of herkogamy; Table 1, Fig. 3). The variation pattern is consistent among populations: flowers belonging to the Castro population exhibited the largest sexual organs, whereas those in Grixoa showed the smallest.

Not surprisingly, all size-related variables showed medium to strong correlations and exertion and herkogamy were correlated as expected with style and corona size. We also found a positive relationship for herkogamy and exertion, reflecting the fact that both variables increased with style length (Table 2).
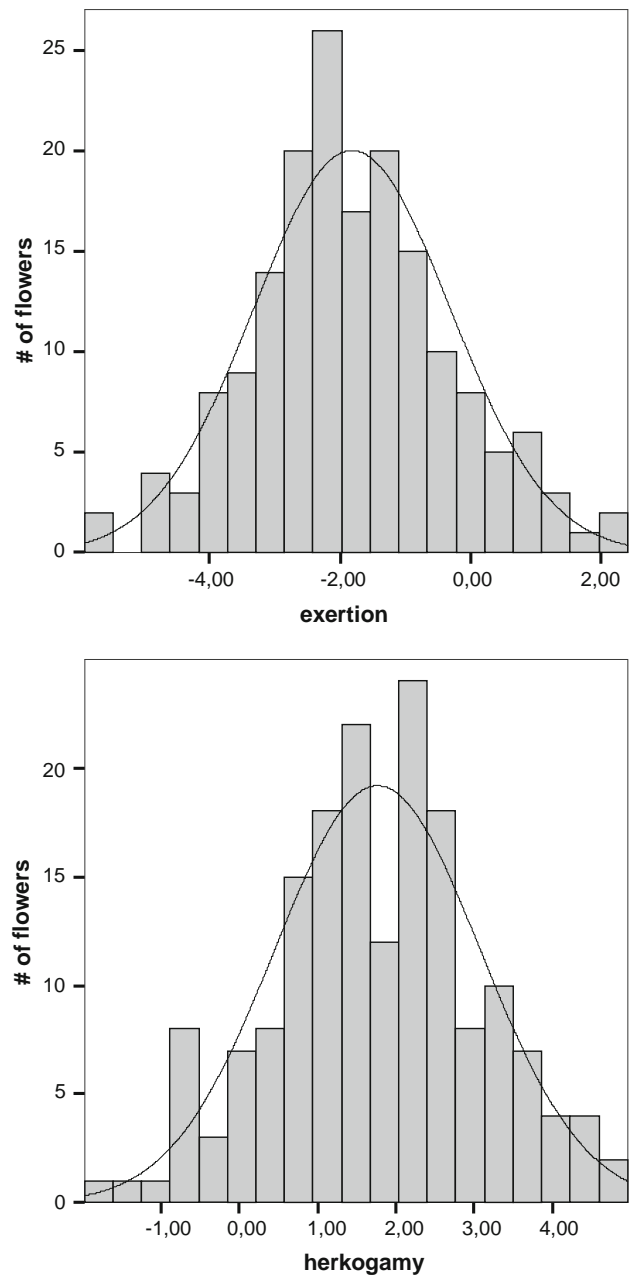

Fig. 2 Histograms of herkogamy and exertion (see text for details), where both variables can be seen to be highly variable and show a unimodal distribution

Breeding systems

Mean fruit set did not differ significantly between populations and since the Population effect presented a P value higher than 0.25, it was omitted from the final model. As can be seen in Fig. 4(a, b), pollination treatment exerted a significant effect on the mean fruit set (df = 2, v^2 = 15.58, P $\searrow$ 0.0001), although a marginally significant interaction effect (df = 2, v^2 = 5.21, P = 0.074) suggests that these

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Floral trait	Mean	Minimum	Maximum	SD	CV (%)	$F_{(3,169)}$	P
Corona length	16.79	11.95	23.13	2.03	12.12	2.08	0.1050
Corona diameter	6.67	4.76	9.86	0.91	13.66	3.37	0.0199
Corona aperture	5.80	3.22	9.57	1.26	21.73	6.45	0.0004
Stamen length	13.21	8.78	18.21	1.88	14.27	23.24	**0.0000**
Filament length	6.37	3.22	10.11	1.44	22.57	24.52	**0.0000**
Anther length	6.84	4.41	10.12	1.13	16.51	5.78	**0.0009**
Floral trait	**Mean**	**Minimum**	**Maximum**	**SD**	**CV (%)**	$F_{(3,168)}$	**P**
Style length	14.95	11.03	22.26	1.94	12.95	4.87	**0.0028**
Stigmatic exertion	−1.82	−5.92	2.40	1.51	82.78	1.64	0.1824
Herkogamy	1.77	−1.98	4.96	1.31	74.44	8.58	**0.0000**

Table 1 Main descriptive statistics and population variation of floral traits (N = 173, measure units: mm)

Population effect was estimated through univariate ANOVAs. Significant values, after applying Bonferroni's sequential correction, are bold typed. The high value of the coefficient of variation presented by both herkogamy as well as stigmatic exertion is noteworthy

differences in pollination treatments may vary depending on the population considered.

Seed production per fruit ranged from 1 to 39 (mean = 12.46, SD = 10.89), showing a pattern of variation similar to that of fructification rates (Fig. 5). After eliminating the Population effect from the model (P ⌐ 0.25), the only significant effect was Pollination Treatment ($df_{PolTreat}$ = 2, df_{error} = 32, F = 8.05, P = 0.001; Population 9 Pollination Treatment interaction: $df_{Pop*PolTreat}$ = 2, df_{error} = 32, F = 2.56, P = 0.093). As shown in Fig. 5, the two autogamous treatments did not reveal significant differences (a priori contrast, t = −0.545, P = 0.453), while the number of seeds produced by fruits pollinated with xenogamous pollen was greater than the number resulting from facilitated autogamy (a priori contrast, t = −1.715, P = 0.002).

Average seed weight and the relationship between the number of seeds per fruit and their weight varied between populations and between pollination treatments (Table 3). In the latter case, the differences depended on the value of the covariate, "number of seeds per fruit", with spontaneous autogamy showing a sharper trade-off between seed number and mean weight (Fig. 6).

Stigmatic exertion and approach herkogamy

None of the individuals examined in the lab showed any variation in herkogamy over the flowering period (df_{meas} = 6, df_{error} = 54.249, F = 0.774, P = 0.593). All the flowers marked in the field did set fruit. The seed production per fruit was not related to the degree of stigmatic exertion, corona size, or level of herkogamy, as indicated by the poor fit of the model (df_{model} = 3, $df_{residual}$ = 17, F = 0.656, P = 0.590) and the lack of significance for any of the effects (herkogamy, slope = −0.114, t = 0.247, P = 0.808; exertion, slope = 0.190, t = 1.131, P = 0.274 and corona slope = 0.222, t = 1.274, P = 0.220). The degree of herkogamy had a

marginally significant and positive effect on the mean seed weight per fruit (slope = 0.966, t = 1.781, P = 0.091; model R^2 = 0.143, df_{model} = 1, $df_{residual}$ = 19, F = 3.173, P = 0.091). Seed weight under natural pollination conditions was not significantly affected by exertion, corona length, or number of seeds (either aborted or ripe) (all P ⌐ 0.25).

Discussion

The results show that N. cyclamineus is a self-compatible, monomorphic species, both of which are ancestral characteristics within the genus Narcissus (Graham and Barrett 2004; Barrett and Harder 2005; Barrett and Hodgins 2006). Furthermore, it exhibits a considerable variation in the relative position of the style with respect to the stamens (approach herkogamy). While the coefficients of variation of the floral structures are relatively low (i.e. sizes of sterile structures less than 25% in all cases), those related to herkogamic and stigmatic exertion are much higher. Stylar size and herkogamic values are similar to those found for the monomorphic Narcissi of the Apodanthi section (Pérez et al. 2004). Although part of this variation can be attributed to ontogenic changes in flower traits, the effect on fitness surrogates found here would suggest that at least part of the variation measured is due to inter-individual differences in herkogamy and stigmatic exertion. Likewise, in N. longispathus, another monomorphic species with variation in herkogamy levels, temporal change in the separation of sexual organs throughout the flower's life is not significant (Medrano et al. 2005). In N. cyclamineus, the previous monitoring of 600 flowers throughout their whole life cycle pointed to a lack of ontogenic changes in exertion (personal observation). In the present work, we have confirmed a lack of ontogenic changes in the degree of herkogamy for this species, by monitoring the flowers individually in the lab.

Fig. 3 Variation between populations in variables studied. Remarkably, the four populations distribute along a flower size and herkogamy gradient. Different letters indicate a significant difference (Tukey post-hoc test)
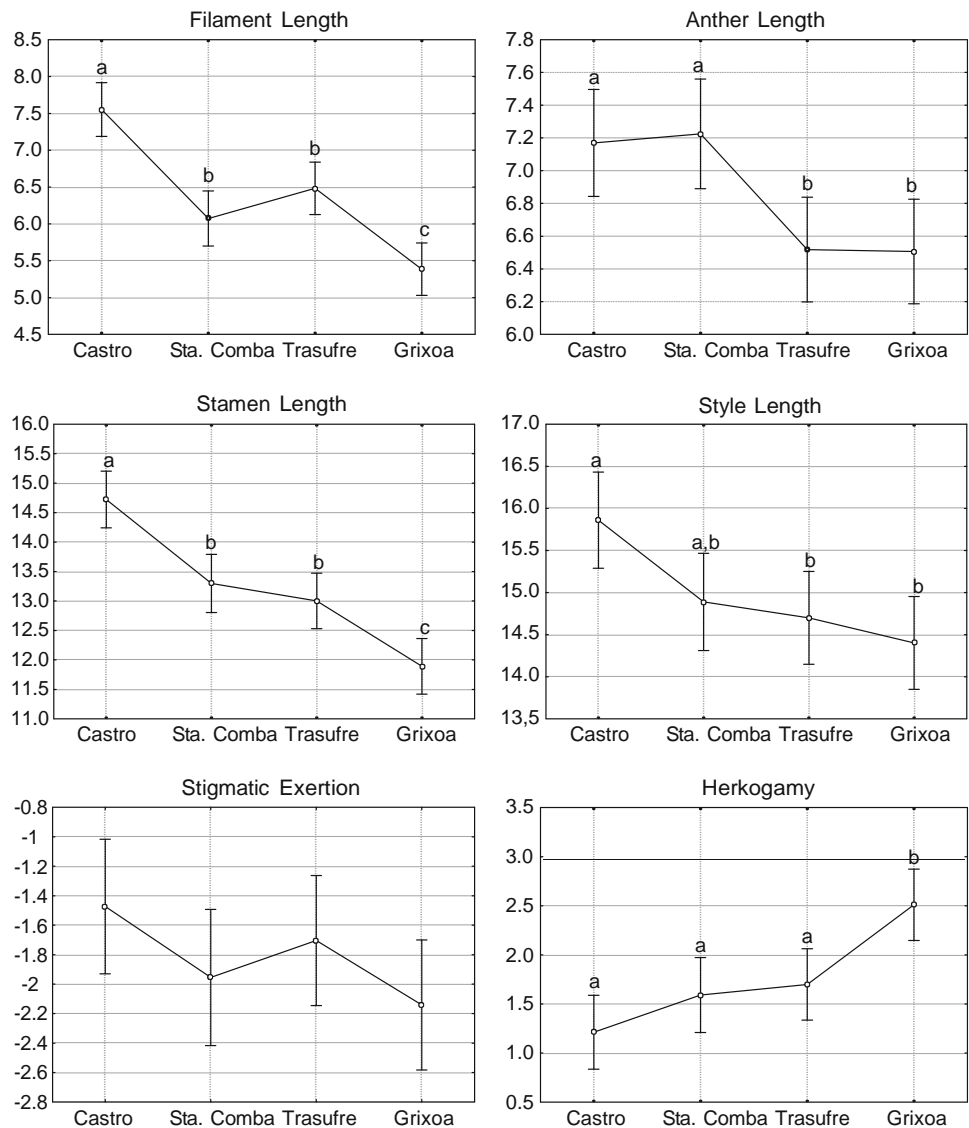
Nearly all the monomorphic characteristics studied, with the exception of flower size, exhibited variation between populations. In the case of herkogamy, this variation is a common phenomenon in many different taxa (see for example Holtsford and Ellstrand 1992; Carr and Fenster 1994; Carr et al. 1997). As Fig. 2 shows, approach herkogamy is more marked in populations with smaller sexual organs, although the limited number of populations studied would preclude generalization. This relationship is not present at the plant level; moreover, the larger the flower, the higher the herkogamy and stigmatic exertion (results not shown).

Breeding systems, herkogamy, and stigmatic exertion

In contrast to Bateman's indications (1954), our results show that N. cyclamineus is a self-compatible species able to produce seeds in both facilitated and spontaneous self-pollination conditions.

Analyses carried out on fruit set and seed production point to a possible inbreeding effect on this species. Both the number of ripe fruits and the number of seeds per fruit are higher for outcrossed flowers (xenogamy treatment) than for self-pollinated ones (facilitated autogamy), despite the fact that hand pollination is supposed to avoid pollen limitation effects. Under inbreeding depression, outcrossing mating systems would be selectively favored, although models incorporating the costs and benefits of selfing predict stable mixed mating systems in natural populations (Lloyd and Schoen 1992; Goodwillie et al. 2005).

We found no relation between herkogamy and the number of seeds produced per fruit. Although our data on herkogamy do not allow us to assess the rate of self/

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	Corona Diameter	Corona Aperture	Stamen Length	Filament Length	Anther Length	Style Length	Exertion	Herkogamy
Corona Length	**,609**	**,368**	**,628**	**,416**	**,519**	**,711**	**-,430**	,170
Corona Diameter		**,617**	**,459**	**,256**	**,441**	**,487**	-,185	,106
Corona Aperture			**,310**	,184	**,283**	**,261**	-,139	-,012
Stamen Length				**,801**	**,649**	**,761**	,142	**-,293**
Filament Length					,065	**,572**	,189	**-,283**
Anther Length						**,532**	-,006	-,125
Style Length							**,328**	**,398**
Exertion								**,283**
Herkogamy								

Significant values after applying Bonferroni's sequential correction are bold typed

outcross pollen arriving to experimental flowers, these results suggest that herkogamy does not increase the amount of outcross pollen.
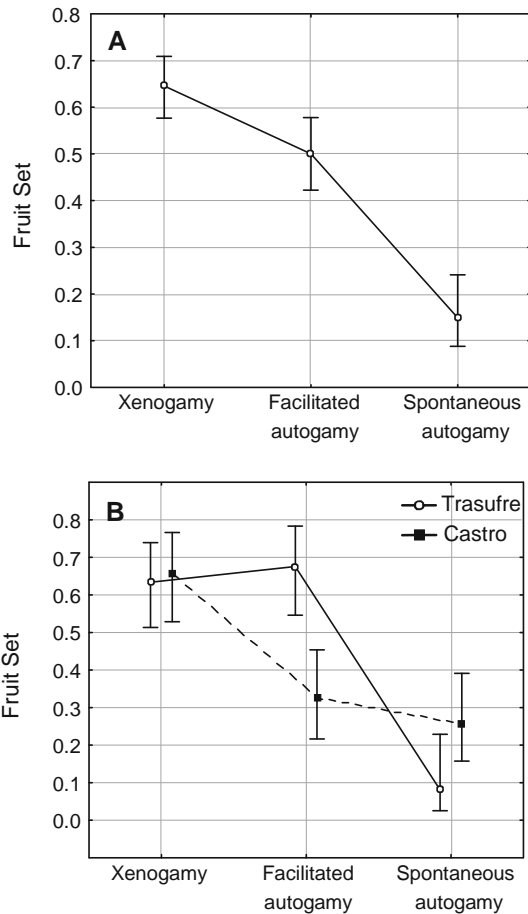

Fig. 4 Estimated grand means of the probability of fruit set for the three pollination treatments (a) and estimated means for each treatment at each of the sites (b)

Our analyses suggest that pollen origin has an important effect on the relationship between seed weight and number. While spontaneous autogamy resulted in bigger seeds only for low seed numbers (\9 seeds), facilitated autogamy resulted in bigger seeds for larger seed numbers, which are usually found in the field (from 20.25 to 34.44). Although fruits from spontaneous autogamy always yielded low seed numbers, this change in the trade-off between seed number and weight deserves further attention.

Plants with a greater separation between stigmas and style seem to produce heavier seeds than those presenting
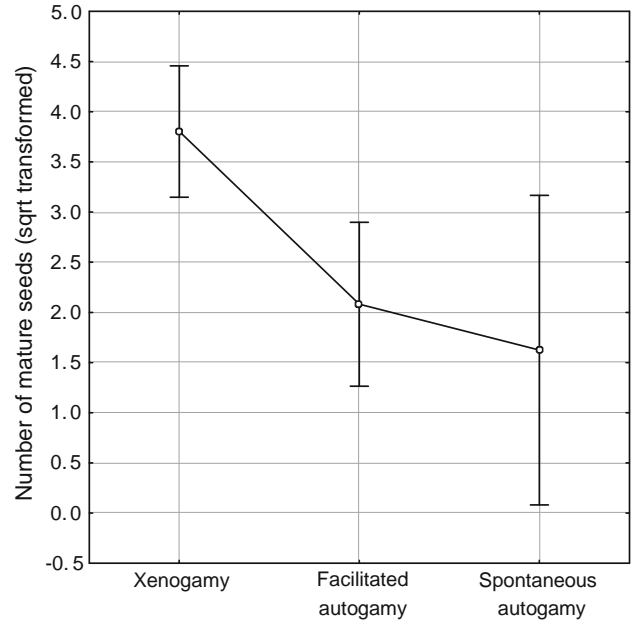

Fig. 5 Estimated grand means of the number of mature seeds for the three pollination treatments. Outcrossing flowers produced more seeds than self-pollinated ones

Table 3 Analysis of the effects of population, pollination treatment and number of seeds on mean seed weight

	SS	df	MS	F	P
Population (P)	16.065	1	16.07	6.484	0.017
Treatment (T)	24.945	2	12.47	5.034	0.014
Seeds (S)[a]	32.984	1	32.98	13.313	0.001
P ✿ S	11.658	1	11.66	4.705	0.039
T ✿ S	21.144	2	10.57	4.267	0.025
P ✿ T ✿ S	13.447	2	6.72	2.714	0.085
Error	64.417	26	2.47		

The population ✿ treatment effect was eliminated from the model due to a P value higher than 0.25

[a] Seeds, number of mature seeds, square root transformed

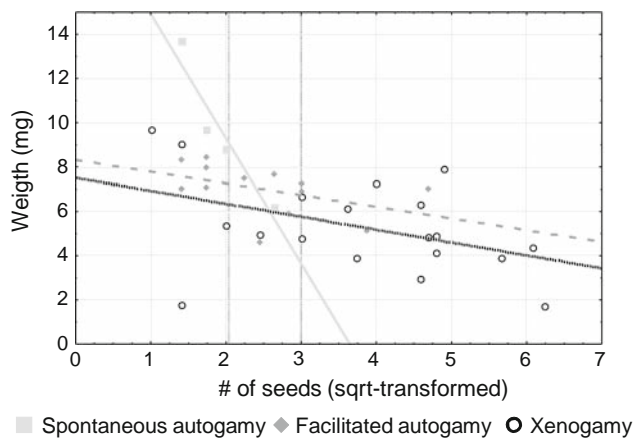■ Spontaneous autogamy ◆ Facilitated autogamy ○ Xenogamy

Fig. 6 Simple slopes of the relation between the number of mature seeds per fruit (square root transformed) and the average seed weight per fruit. Dotted line: xenogamy, dashed line: induced autogamy, solid line: spontaneous autogamy. Vertical dashed lines indicate the region of significance for the comparison between the two types of autogamy; when the square root of the number of seeds falls out of this region, the difference between the two treatments is statistically significant (Johnson–Neyman analysis; no region of significance for the remaining variable pairs)

more exerted stigmas, although this relationship was only marginally significant. One possible explanation could be that a higher degree of herkogamy diminishes rates of pollination with the plant's own pollen and produces better quality seeds. Herkogamy has been deemed a mechanism for reducing self-fecundation (see Barrett 2002 and references in this work), a strong positive link that has often been found between the degree of herkogamy and outcrossing rates (Holtsford and Ellstrand 1992; Belaoussoff and Shore 1995; Karron et al. 1997; Herlihy and Eckert 2004). However, our results on the breeding system of N. cyclamineus do not support this hypothesis, as there are no significant differences in seed weight between facilitated autogamy and xenogamy. Medrano et al. (2005)

recently reported that a positive correlation between herkogamy and outcrossing rate is not a general pattern in the genus Narcissus. An alternative hypothesis postulated that a different quality of pollen would arrive at the stigma as a function of herkogamy rate, mediated by pollinator behaviour. Although our results do not allow us to evaluate this hypothesis, we did find a negative relationship between herkogamy rates and inter-individual variation of seed weight (data not shown), suggesting that pollen quality differences could be related to the effect of herkogamy observed on seed weight.

Contrary to our findings in the breeding system experiment, seed number was not related to seed weight for our naturally pollinated flowers, when analyzing the role of herkogamy. However, we cannot rule out the possibility that bagging the whole scape before floral anthesis might have strongly affected the level of available resources for fruit production and ripening. In fact, our experimental bagged flowers showed a lower fruit set and seed number than unmanipulated flowers monitored over the course of two years in three different populations (unpublished data).

In conclusion, our results reveal a considerable variation in floral morphology as well as in the characteristics associated with sexual organs (herkogamy and stigmatic exertion) between populations of N. cyclamineus. The relative position of the stigmas and anthers has a significant effect on female reproductive success, which highlights the need to analyze the reproductive consequences of continuous variations in the monomorphic species of the genus Narcissus.

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