

# The avoidance of self-interference in the endemic daffodil *Narcissus cyclamineus* (Amaryllidaceae)

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**Abstract** Hermaphrodite flowers usually possess floral traits to avoid the negative effects derived from inbreeding depression and/or self-interference between pollen export and reception, both acting as the main selective pressures on those floral traits. The avoidance of self-interference is widely accepted as the primary force promoting the separation between sexes within the flowers in time (dichogamy) and/or space (herkogamy) for self-incompatible species, which are already protected from the negative effects of inbreeding depression by the incompatibility system. Different degrees of incompatibility, herkogamy, and dichogamy have been reported for the genus *Narcissus*. However, the only mechanism for the separation of sexes reported up to date for *Narcissus cyclamineus* is herkogamy, while the presence of dichogamy and the type of incompatibility in this species remain uncertain. In this study, we analyze the patterns of sexual reproduction in *N. cyclamineus* to ascertain whether there is any selective pressure favouring sexual segregation or its maintenance and their

mechanisms. *N. cyclamineus* is self-incompatible and dichogamy can be rejected for this species. Even though the species is self-incompatible, when cross-pollination is preceded by self-pollination the number of ovules available for legitimate crosses is diminished (ovule discounting). Pollinators are scarce during the flowering period, resulting in pollen limitation. It is suggested that both the scarcity of pollinators and ovule discounting may be acting synergically to promote herkogamy or its maintenance in this species.

**Keywords** Herkogamy · Dichogamy · *Narcissus cyclamineus* · Self-incompatibility · Self-interference · Ovule discounting

## Introduction

The co-location of female and male function within the same floral structure is a common feature in flowering plants. There are some advantages derived from this hermaphroditic condition, namely the economy of resources invested in attracting pollinators and increasing probability of selfing when mates or pollinators are scarce (Charlesworth and Charlesworth 1987; Lloyd 1987). But, hermaphroditism may be inconvenient because it may increase inbreeding depression and/or self-interference (interference between the pollen-export and the pollen-reception functions) (Lloyd and Webb 1986; Webb and Lloyd 1986; Barrett and Harder 1996). A number of floral traits

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have evolved to prevent those disadvantages, and the spatial separation of stigmas and anthers within the flower (herkogamy) is one of the most frequent (Webb and Lloyd 1986). However, many plant species are known to be self-incompatible and therefore protected from the negative effects of inbreeding by their inability to self-reproduce (e.g. Cesaro et al. 2004); in these cases, the avoidance of the self-interference is the main selective force promoting floral traits related to self-pollination avoidance rather than the prevention of the inbreeding depression (Harder and Barrett 1996; Harder et al. 2000; Barrett 2002, 2003; Medrano et al. 2005).

It is therefore important to assess whether plant species are self-compatible or self-incompatible to understand the evolutionary significance of the traits associated with avoiding self-pollination in flowering plants. Self-incompatibility has been defined as the “partial or complete inability of a functionally bisexual seed plant to produce zygotes after self-pollination” (Becerra and Lloyd 1992). Importantly, those authors acknowledge that self-incompatibility seldom results in the complete loss of all ovules, and should be characterized quantitatively. Seavey and Bawa (1986) acknowledge the difference between “self-incompatibility” (at the level of the stigma or style), and “late-acting incompatibility,” when “the barriers are in the ovary.” In this context, *ovule discounting* (i.e., the loss of ovules after self-pollination) in *Narcissus* has been considered a late-acting self-incompatibility mechanism since it occurs before the pollen tube entry into the ovary (Sage et al. 1999), which would involve a still unknown long-distance signaling mechanism between pollen tubes, ovary, and ovules (Sage et al. 1999; Barrett 2002; Cesaro et al. 2004). The mechanism of ovule discounting may vary among lineages, and some cases have even been documented of a reversible effect of the interference: for *Brassica nigra*, Ockendon and Currah (1977) reported an inhibitory effect of self-pollen on the stigma which decreases over time although such results could not be confirmed for other Brassicaceae (*Raphanus raphanistrum*, Koelling and Karoly 2007). The existence of ovule discounting in polymorphic species of the genus *Narcissus* has been also documented (e.g., Barrett et al. 1997; Sage et al. 1999; Arroyo et al. 2002; Cesaro et al. 2004), but to our knowledge it remains to be proven in a monomorphic approach-herkogamous species (with a stigma protruding further than the

anthers); this state was inferred to be ancestral for the genus (Graham and Barrett 2004).

*Narcissus cyclamineus* is a monomorphic approach-herkogamous endemic species from the northwestern Iberian Peninsula. The type of compatibility in this species is a subject of controversy since it has been characterized not only as self-incompatible (Bateman 1954) but also as self-compatible (Larrinaga et al. 2009). In order to understand the significance of herkogamy for this species, first of all, we intend to confirm or rule out whether there are other mechanisms to prevent self-pollination, namely dichogamy. To this end, an accurate assessment of temporal patterns of the sexual functions within the flowers must be accomplished to know if a temporal between-sex separation is also present as for other species of the genus (e.g., Cesaro et al. 2004); then, we will study the morphological floral traits of *N. cyclamineus* to precisely characterize herkogamy and the variability of the floral shape as phenotypic floral integration. In order to assess whether the function of herkogamy in this species is to prevent inbreeding, self-interference, or both, we will study whether the species is self-compatible or self-incompatible. Since self-incompatibility was confirmed, we will then determine the existence of ovule discounting as described for other polymorphic species of the genus. Finally, we assess the main patterns of the pollination process to determine the importance of the pollen flow on the sexual reproduction for this species.

## Methods

### Study species and site

*Narcissus cyclamineus* DC. (Amaryllidaceae) is a tall (20–30 cm), unifloral, bulbous herb with yellow flowers. Flowering starts in mid-February and lasts until the end of March, and fruits mature until June–July, when the capsules open to allow seed release. The single flower of each plant is protected by a spathe and consists of six deflexed tepals and a corona with a crenate–serrate margins. The corona encloses six equal-sized stamens and a stigma positioned above the anthers (approach-herkogamy). Its area of distribution is restricted to the north-western part of the Iberian Peninsula, where it grows close to river banks at low altitude (Pino Pérez et al. 2005).

The studied population is located near the village of Zamáns (Vigo—Spain, 42°09′28″N 8°41′30″W), in meadows at the confluence of the Amial and Zamans streams, comprising an area of about 385 m<sup>2</sup>. Annual mean temperature and precipitation are 13.6 °C and 1,909 mm, respectively, [data from the nearest recording meteorological station, at about 8.5 km from the study site, INM (Instituto Nacional de Meteorología) 2004]. The number of flowering individuals in this population is about 6,900. Since *N. cyclamineus* is an endangered species, all experiments carried out in this study were non-destructive; bulbs gathered for the experiments conducted in the laboratory were planted back in the field once the experiments were concluded.

### Floral morphology

The flowers from 112 randomly selected individuals were collected and kept in ethanol (70 %) until being processed. Each flower was photographed in the laboratory against calibrated graph paper, before and after dissection. We measured the following floral variables on the photos: corona length, tepal length, corona aperture, corona diameter, style length, stigma diameter, tube length, ovary length, stamen length, anther length, and stem angle (Fig. 1). All measurements were taken by the image analyzer software *Analysis 5.0* (Soft imaging system GmbH 1999). We also calculated the “herkogamy distance” (difference between the style and stamens lengths) and the “stigma exertion” (difference between the style and tube lengths) for each flower.

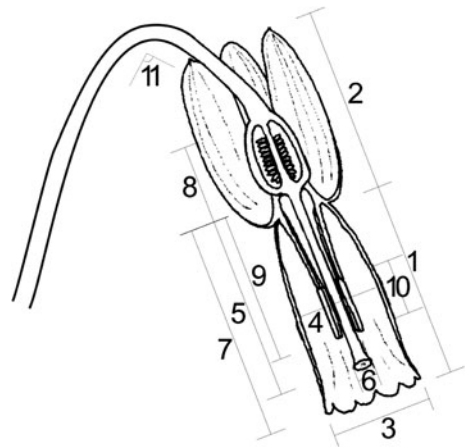
### Temporal patterns of the floral features

#### *Floral lifespan*

To determine the flower longevity, 22 floral buds were randomly selected, marked, and monitored daily until the corollas withered.

#### *Pattern of nectar production*

The production of nectar was evaluated in 4 different age classes: 1, 5, 9, and 13 days after anthesis. For each class, 15 flowers were bagged in the field 24 h



**Fig. 1** Schematic representation of a flower of *N. cyclamineus*, showing the measures taken for the present study, modified from Larrinaga et al. (2009). Variable numbers correspond to: 1 corona length; 2 tepal length; 3 corona aperture; 4 corona diameter; 5 style length; 6 stigma diameter; 7 tube length; 8 ovary length; 9 stamen length; 10 anther length; 11 stem angle

before nectar extraction. We used nylon mesh bags; these bags have been regularly used in this type of study (e.g., Navarro 1999; Ferrero et al. 2009) as they are known to have little influence on nectar production (Wyatt et al. 1992). The nectar volume was measured using capillary micropipettes and the sugar concentration was determined using a portable refractometer as the percentage by weight. The amount of sugar produced by each flower was estimated according to Cruden and Hermann (1983).

#### *Pollen release and fertility*

To assess the pattern of pollen liberation, 22 floral buds were randomly selected (different from those used for floral lifespan determination) and marked in the field before anthesis, and monitored daily during the lifespan of the flower. Every day a glass slide was placed under the floral tube to collect the pollen liberated by gently shaking the flower. To evaluate the ability of the pollen to germinate, one hundred pollen grains per flower (when available) were randomly selected from those collected each day and placed in a petri dish on a sterile solid medium containing 10 % sucrose, this being the concentration which gave the highest germination rate in preliminary tests with 0, 10, 20, 30, and 40 % sucrose. Pollen grains were

incubated for 4 h and germination counts were done under a stereomicroscope.

### Female function

To assess the pistil receptivity during the flower's lifespan, hand-pollinations were performed on flowers of nine different age classes: 1, 3, 5, 7, 9, 11, 13, 15, and 17 days for each class. Floral buds were emasculated and then bagged before anthesis; after anthesis, 10–13 flowers per class were hand pollinated with a mixture of fresh pollen collected from 10 different individuals, and bagged again until withered. Fruits were collected after 4 weeks, and the seed to ovule ratio was estimated for each age class.

### Breeding system and ovule discounting

Six treatments were carried out in the field to assess the breeding system in *N. cyclamineus*. Twelve flowers were randomly assigned to each treatment, some of which were lost because of trampling by ungulates: (1) pollen supplementation (non-bagged flowers/hand pollinated with xenogamous pollen,  $n = 12$ ); (2) forced xenogamy (bagged flowers/hand pollinated with xenogamous pollen;  $n = 11$ ); (3) spontaneous xenogamy (non-bagged flowers/emasculated before anthesis/no hand pollination,  $n = 12$ ); (4) forced autogamy (bagged flowers/hand pollinated with autogamous pollen,  $n = 12$ ); (5) spontaneous autogamy (bagged flowers/no hand pollination;  $n = 11$ ); and (6) untreated control ( $n = 9$ ). After 4 weeks, the fruits were collected for the evaluation of the seed-to-ovule ratio (S/O henceforth) and seed weight measurement. Seeds were dried for 15 days in the lab at room temperature and stored in a desiccator with silica gel for at least 24 h before weighing.

The *selfing rate* was calculated as  $S = (P_x - P_o) / (P_x - P_s)$ , where  $P_x$  is the S/O after hand cross-pollination,  $P_s$  after hand self-pollination, and  $P_o$  under natural conditions (after Charlesworth and Charlesworth 1987). Since self-incompatibility is a quantitative trait (Becerra and Lloyd 1992), it only can be correctly ascertained by quantification. The *self-compatibility* index (SCI) was calculated following Lloyd and Schoen (1992), as the relation between the mean S/O from facilitated autogamy and the mean S/O from facilitated xenogamy treatments. The reproductive success limitation due to insufficient pollen receipt

was estimated with the *percentage of pollination limitation* (PPL) index as  $PPL = [100 \times (P_s - C)] / P_s$ , where  $P_s$  is the S/O of pollen supplemented plants and  $C$  is the S/O of control plants (Jules and Rathcke 1999).

To evaluate whether the S/O decreases as a result of self-pollen interference, i.e., the possibility of ovule discounting, 50 bulbs were transplanted to the laboratory before the flowering period and the following treatments were performed after floral dehiscence (all bulbs produced flowers; 10 flowers were randomly assigned to each treatment): (1) autogamy—hand pollination with self-pollen; (2) xenogamy—floral buds were emasculated and hand pollinated with a mixture of pollen from 10 different flowers after dehiscence (treatments 1 and 2 are the controls to the following); (3) autogamy followed by xenogamy after 12 h; (4) autogamy followed by xenogamy after 24 h; (5) autogamy followed by xenogamy after 48 h.

### Pollination ecology

To determine the floral visitor's assemblage, direct observations were performed from 2005 to 2008. Surveys were performed during the flowering peak in several randomly selected areas of approximately  $2 \times 2$  m (approximate plant density  $20 \text{ plants m}^{-1}$ ) and the observer was positioned at approximately 1 m apart from the area to avoid disturbing the pollinators. Insect visits were recorded during sessions of 15 min of surveillance at different hours of the day. A total of 35 h of census were performed, distributed in similar times of observation per year. For each visit, the visitor species was recorded.

In addition, to evaluate the relative contribution of diurnal versus nocturnal pollinators, two groups of flowers in the field were protected from pollinators during different hours in the day. Sixteen flowers were randomly assigned to each treatment, some of which were lost because of trampling by ungulates: (1) floral buds were bagged and after dehiscence the bags were removed every morning during the flowers' lifespan to allow its exposure from dawn to dusk ( $n = 16$ ); (2) floral buds were bagged and after dehiscence the bags were removed every night allowing the exposure of the flowers from dusk to dawn ( $n = 14$ ); finally (3) a group of flowers were just marked as control ( $n = 9$ ).

We also estimated the pattern of pollen flow using dyed powder as pollen substitute. A group of 15

flowers within an area of about 30 cm in diameter were selected as focal. The flowers were labeled and all anthers of all flowers were powdered with orange fluorescent powder (Kearns and Inouye 1993). This focal group was the center of 13 different circular distance-classes (radius 0–1; 1–2; 2–3; 3–4; 4–5; 5–6; 6–7; 7–8; 8–9; 9–10; 10–25; 25–50; >50 m, respectively, for each class); seven days later 15 flowers (if available) were randomly taken from each class, and stored frozen ( $-20\text{ }^{\circ}\text{C}$ ) until analyzed. The flowers were analyzed using a stereomicroscope under UV light (365 nm) to look for dyed powder; the presence of powder and its position in the flower was recorded.

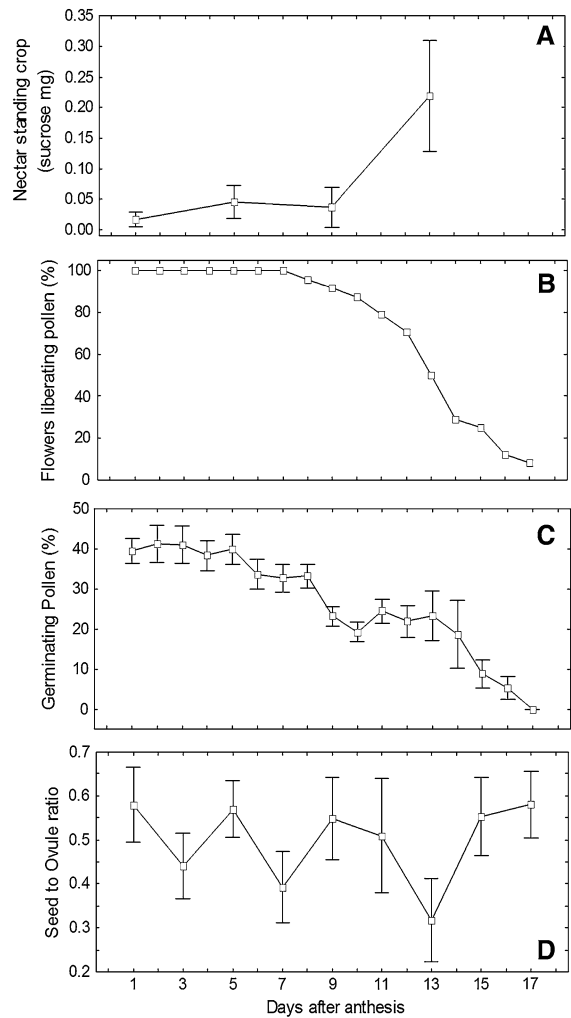
### Data analysis

The index of phenotypic integration (INT) was calculated with the morphometric data of the flowers as proposed by Wagner (after Ordano et al. 2008). This index measures the variance among the eigenvalues of the phenotypic correlation matrix. A high variance among eigenvalues indicates a high degree of integration because most phenotypic variation is accounted for by the first principal component. The index of phenotypic integration can be expressed as the percentage of the maximum variance expected.

The results of pollen germination (%) and female receptivity (S/O) were compared between days with one-way ANOVA; the results of the breeding systems and ovule-discounting experiments (both S/O) were also compared by one-way ANOVA. All data were arcsin transformed as recommended for proportion variables (Zar 2009), and homogeneity of variances was verified by the Cochran's *C* test. ANOVA's post hoc comparisons were evaluated by the Tukey's test.

Nectar standing crop between days and seed weight among breeding system treatments were compared by the Kruskal–Wallis non-parametric test since the data did not fulfill the homoscedasticity assumption of ANOVA even after transformation. For the same reason, the results of the day versus night pollination experiment were pairwise compared by the Mann–Witney non-parametric test; the probability was considered after the Bonferroni correction for multiple comparisons.

Even though the parametric tests were performed on transformed data, Figs. 2, 3, 4, and 5 are showing the actual (untransformed) values for illustrative



**Fig. 2** Variation of some floral characteristics along the floral lifespan in *N. cyclamineus*: **a** nectar standing crop as sucrose content (mg); **b** percentage of flowers releasing pollen; **c** percentage of pollen grain germination; and **d** receptivity of the pistils measured as seed to ovule ratio after controlled hand pollination (mean values  $\pm$  standard errors, except for **b**)

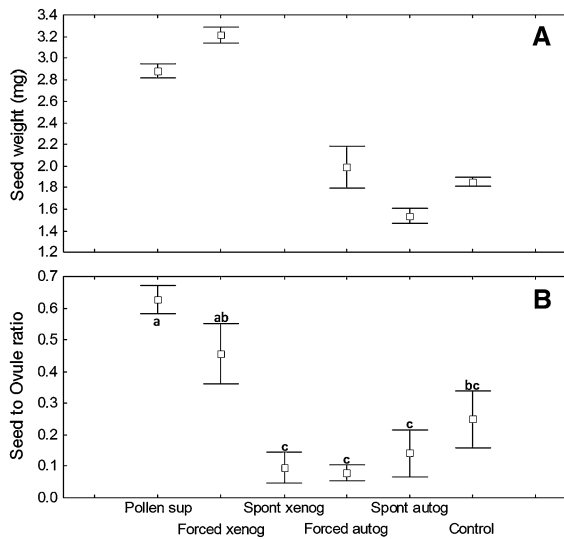
purposes. Differences were considered significant when  $P < 0.05$ . All analyses were performed by the STATISTICA 7.0 (Statsoft Inc.) software package.

## Results

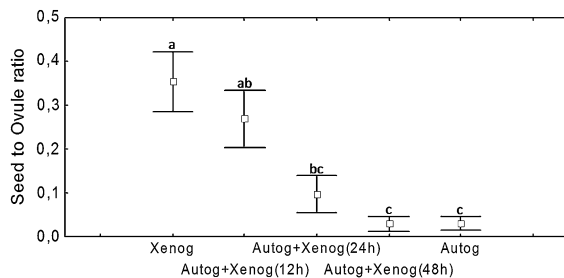
### Floral morphology

The main descriptors of the morphometric floral variables are shown in Table 1. Most of the variability of the dataset is gathered by the first principal





**Fig. 3** Mean seed weight (a) and mean seed to ovule ratio (b) after the breeding treatments in flowers of *N. cyclamineus*: pollen supplementation, forced and spontaneous xenogamy, forced and spontaneous autogamy, and control (mean values  $\pm$  SE). Different letters stand for significant differences in the ANOVA post hoc test

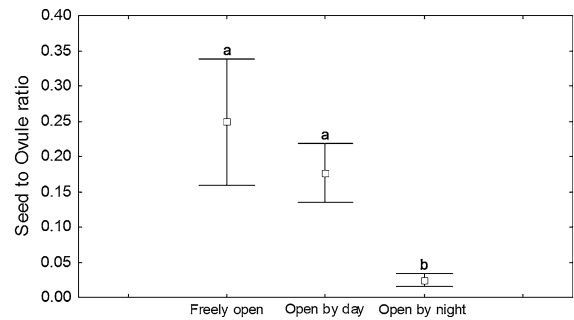


**Fig. 4** Results of the experiment to assess the ovule discounting due to self-pollination in *N. cyclamineus*: control, forced xenogamy, forced autogamy followed by forced xenogamy after 12, 24, and 48 h, and forced autogamy only (mean  $\pm$  SE). Different letters stand for significant differences in the ANOVA post hoc test

component (57.8 %) and the phenotypical integration for this species is  $INT = 29.73$  %.

#### Temporal patterns of floral features

Floral lifespan of *N. cyclamineus* is long (mean  $18.55 \pm 2.92$  days). Nectar production was minimal at anthesis and highest toward the end of the flower lifespan (Fig. 2a), although the differences between days were not statistical significant (Kruskal–Wallis  $H = 3.61$ ,  $P = 0.31$ ). The recorded sugar concentration of the nectar ranged between 1.00 and 1.08 mg/ $\mu$ l.



**Fig. 5** Results of the treatments performed to assess the differential pollination success between day and night in *N. cyclamineus* flowers (means  $\pm$  SE). Different letters stand for significant between-group differences

Pollen release was highest during the first seven days after dehiscence, and decreased linearly after the seventh day (Fig. 2b). Pollen germination was constantly under 50 % (Fig. 2c) with the highest values at anthesis and decreasing gradually after the fifth day ( $F = 6.65$ ,  $df = 16$ ,  $P < 0.001$ ); although pollen germination in vitro may be lower than in vivo (Taylor and Helper 1997), this result is meaningful for among-days comparative purposes.

Mean pistil receptivity ranges from 0.3 to 0.6, and is almost constant during all the lifespan of the flower (Fig. 2d). We have not found any differences among age classes in the ability of flowers to set seed ( $F = 0.34$ ,  $df = 8$ ,  $P = 0.24$ ).

#### Breeding systems and ovule discounting

Results from the breeding treatments are shown in Fig. 3. There were differences in the S/O ratio among pollination treatments ( $F = 10.94$ ,  $df = 4$ ,  $P < 0.001$ ). According to the post hoc analysis, two different groups of treatments could be recognized; on one side, supplementary and forced xenogamous pollination which significantly improved the S/O ratio; and on the other, spontaneous xenogamy, forced autogamy, spontaneous autogamy, and field control although the latter with slightly higher values (Fig. 3b). A similar result was attained when considering the seed weight as the fitness measure (Kruskal–Wallis  $H = 201.56$ ,  $P < 0.001$ , Fig. 3a), confirming the treatment's effect.

*N. cyclamineus* is a self-incompatible species according to the results of the selfing rate ( $S = 0.08$ ) and the self-compatibility index ( $SCI = 0.17$ ). This

**Table 1** Measurements of the floral traits of *N. cyclamineus* ( $n = 112$ )

Floral trait (mm)	Code	Mean	Minimum	Maximum	SD	CV (%)
Corona length	1	17.44	13.01	25.26	2.13	12.21
Tepal length	2	17.63	12.74	24.23	2.42	13.71
Corona aperture	3	7.78	4.96	11.16	1.12	14.38
Corona diameter	4	5.53	3.62	7.87	0.72	12.99
Style length	5	15.99	13.04	20.46	1.67	10.44
Stigma diameter	6	1.92	1.16	2.85	0.37	19.19
Tube length	7	18.76	13.97	24.91	2.12	11.28
Ovary length	8	7.34	4.18	14.39	2.05	27.92
Herkogamy distance (style length – stamen length) and stigma exertion (style length – tube length) were calculated from the data measured. Code numbers as for Fig. 1	9	13.14	9.53	17.87	1.40	10.69
	10	4.33	1.77	7.45	0.83	19.14
	11	60.47	15.25	124.63	24.05	39.77
Herkogamy	–	2.35	0.12	5.65	1.00	42.57
Stigma exertion	–	–2.77	–5.94	–0.19	1.35	48.78

population also suffer a high pollen limitation (PPL = 27.06 %), which is illustrated as the difference between the treatments of “pollen supplementation” and “control” in Fig. 3b.

The ovule discounting after self-pollination was significant ( $F = 7.46$ ,  $df = 4$ ,  $P < 0.001$ ), but only when there was no input of xenogamous pollen in the first 24 h after the self-pollination (Fig. 4). If xenogamous pollen reached the stigma within that time, the number of ovules setting seed was not different from the cross-pollinated flowers.

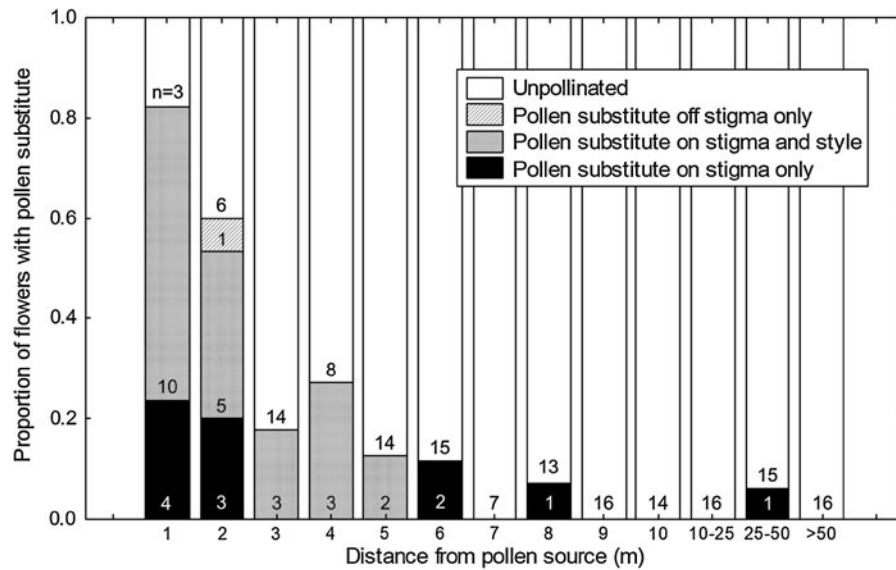
### Pollination

Visits by pollinators were infrequent: in 35 h of survey, only 3 pollinators' species were recorded: *Anthophora* sp. (3 individuals visiting 10 flowers), *Bombus terrestris* (2 individuals visiting 11 flowers), and one unidentified syrphid fly (2 individuals visiting 7 flowers). All of them are generalist pollinators, and their presence was evenly recorded during all the years of this study. Pollination of *N. cyclamineus* was mainly diurnal (Fig. 5) with the highest values of S/O in the flowers open during the day and significantly lower in the flowers open only at night (Mann–Whitney  $Z = 2.373$ ,  $P = 0.03$  for “freely open vs. night”,  $Z = 3.39$ ,  $P < 0.001$  for “day vs. night”).

The pollen substitute was dispersed mostly within a radius of 6 m around the focal flowers (Fig. 6). In most of the distance intervals, the substitute had reached either the stigma only or stigma and style.

### Discussion

The coexistence of male and female sexual organs in hermaphrodite flowers has promoted the evolution of a number of mating strategies to avoid inbreeding depression, self-interference, or both (Barrett 2002, 2003); but, while self-incompatible species are only affected by self-interference, it is difficult to establish which one of those two selective pressures is acting on self-compatible plants. Therefore, to understand the functional significance of the mating strategies in a certain plant species, it is important to confirm whether that species is self-compatible or self-incompatible. Our results clearly demonstrated that *N. cyclamineus* is a self-incompatible species ( $SCI = 0.17$ , far from the threshold value for self-compatibility of 0.75 according to Lloyd and Schoen 1992). Larrinaga et al. (2009) reported this species as self-compatible because seed production after selfing was greater than zero, but they did not calculate any incompatibility index. Indeed, their estimations of fitness after autogamy were conspicuously lower than after xenogamy, implying that SCI values would also be under the 0.75 threshold, and therefore their populations could be considered as self-incompatible after the Lloyd and Schoen (1992) criterion. Although only appropriate genetic studies would make this clear, these results indicate that avoidance of inbreeding in this species is provided by the incompatibility system so the avoidance self-interference is the main selective pressure acting on the flowers of *N. cyclamineus*.



**Fig. 6** Number of flowers of *N. cyclamineus* with pollen substitute from the focal flowers in each of the 13 classes of distance considered

Self-interference may come about as ovule discounting which involves the embryo sac degeneration after self-pollination (see Barrett 2002 for a thorough description, and Sage et al. 1999 for the most plausible mechanism of ovule discounting in the genus); according to our findings, self-interference in *N. cyclamineus* results in ovule sterility. Ovule discounting has been previously described for other species of the genus (e.g., *N. triandrus*, Sage et al. 1999; Barrett et al. 1997; *N. assoanus*, Cesaro et al. 2004; and *N. papyraceus*, Arroyo et al. 2002), all of them polymorphic. Our findings demonstrate that ovule discounting is also present in monomorphic species of the genus; since monomorphism is considered the ancestral state (e.g., Graham and Barrett 2004), this suggests that ovule discounting is also an ancestral trait which could have contributed to the evolution and maintenance of the heterostylous systems in *Narcissus*, as proposed by Cesaro et al. (2004). Interestingly, the number of ovules “discounted” increased with the time after self-pollination, i.e., when cross-pollination quickly followed selfing, there was no ovule discounting, but it was maximum if cross-pollination was delayed 48 h after selfing. Similar results have been reported for the distylous *N. assoanus* (Cesaro et al. 2004).

Ovule discounting in *N. cyclamineus* constitute, therefore, one strong selective pressure to maintain mating strategies to avoid self-interference. But,

despite that the timing of pollen presentation and stigma receptivity is rarely simultaneous in hermaphroditic flowers (Lloyd and Webb 1986; Sargent et al. 2006), and although dichogamy has been reported for other species of the genus (e.g., Cesaro et al. 2004), our results confirm that there is no temporal gap between pollen release and stigma receptivity, therefore *N. cyclamineus* is not dichogamous. Even though there seems to be a decrease of the male function toward the end of floral lifespan, this is probably a strategy to increase the chances of being fertilized by extending the female-function period (i.e., a mechanism of reproductive assurance, e.g. Rathcke 2003), especially in a situation of pollen limitation as we have documented for *N. cyclamineus*.

Herkogamy is then the only floral trait to avoid self-interference in *N. cyclamineus*, not surprisingly since the presence of herkogamy can make dichogamy unnecessary (Sargent et al. 2006). In view of the importance of herkogamy and taking into account that it is a feature derived from the floral shape, it can be expected that those structures contributing to the floral morphology are highly integrated to maintain that shape. Accordingly, Larrinaga et al. (2009) reported that most of the floral traits on *N. cyclamineus* are highly correlated. Our morphometric data are similar to those reported by Larrinaga et al. (2009), and although our herkogamy values are slightly higher,



that difference is certainly not significant (mean  $\pm$  SD  $1.77 \pm 1.31$  for Larrinaga et al. 2009,  $2.35 \pm 1.00$  in this study,  $F$ -test for homogeneity of variances  $F = 1.3$ ,  $P = 0.14$ ). Accordingly, our results confirm high integration values for *N. cyclamineus*, well above of the mean values for flowering plants reported by Ordano et al. (2008). High values of floral integration were proven to be selected by pollinators (e.g., Anderson and Busch 2006; Pérez-Barrales et al. 2007 for other *Narcissus* sp.) although the universality of the selective role of pollinators on floral integration has been questioned (Ordano et al. 2008). Even in a pollen-limited situation like this, high values of floral integration in *N. cyclamineus* can be attributed to the self-incompatibility system which prevents the relaxation of the floral integration that occurs in self-compatible plants (Anderson and Busch 2006).

The pollen limitation in *N. cyclamineus* (close to 30 %) probably reflects the scarcity of pollinators during its flowering period, i.e., late winter. Although a larger number of observation hours would be desirable for a better characterization of the pollinator community, visitors in this population appear to be fewer than in other *Narcissus* sp. (e.g., Pérez-Barrales et al. 2006). With a high number of ovules per flower (mean  $43.49 \pm 19.70$ ) and a low S/O ratio (mean  $0.25 \pm 0.27$  under natural pollination), *N. cyclamineus* behaves as predicted by the model of Burd (Burd 1995, see also Ashman et al. 2004): according to the model, those plants in environments of stochastic pollination should benefit by being over-supplied with ovules to maximize the number of fertilizations from the unpredictable pollen loads received, following a *bet-hedging* strategy. Pollination is mainly diurnal and cross-pollination occurs mainly among plants usually positioned less than 6 m from each other. Despite there being few visits by pollinators, they seem to be quite efficient since the pollen substitute usually reached either the stigma or stigma and style of the target flowers, but was hardly ever placed only outside the stigma. Interestingly, pollinators appear to be more accurate in the longest distances (classes 8 m and 25–50 m), where the pollen substitute was mostly laid right on the stigma. Therefore, the flowers of *N. cyclamineus* seem to be making the most of the scarce visits of their pollinators, in accordance with the *bet-hedging* hypothesis (Burd 1995, Ashman et al. 2004). This pattern of pollen flow matches the foraging behavior of the pollinators recorded in the area

(e.g., Heinrich 1976; Real 1983). Similar results have been found for other species of the genus (*N. bulbocodium*, Navarro et al. unpublished).

Both pollen limitation and ovule discounting seem to be acting synergically to prevent self-interference, and therefore to maintain herkogamy in *N. cyclamineus*: after self-pollination there is a temporal gap for the plant to be cross-pollinated before losing its ovules due to ovule-discounting, but that temporal gap closes rapidly considering the scarcity of pollinators. Therefore, those plants unprotected by a mechanism to prevent self-pollination (herkogamy) are more exposed to ovule loss in a situation of cross-pollen limitation. In our opinion, this possible synergic interaction deserves further attention.

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