STUDIES

Avoiding sexual interference: herkogamy and dichogamy in style dimorphic flowers of Narcissus broussonetii (Amaryllidaceae)

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Abstract.

Spatial (herkogamy) or temporal (dichogamy) separation of sex organs are mechanisms considered to restrict self-pollination and promote outcrossing. Additionally, avoidance of self-interference is proposed to be the driving force for the evolution of these mechanisms, particularly in self-incompatible species. However, species with anthers and stigmas at different levels may increase the rate of imprecise pollen transfer, resulting in pollen discounting. Non-reciprocal stylar dimorphism has been considered a transitional, unstable stage towards the evolution of reciprocal style dimorphism (distyly), to simultaneously avoid interference and lack of precision. In this study we investigate the spatial and temporal separation of sex organs in a population of the style dimorphic and self-incompatible Narcissus broussonetii and their consequences in the reciprocity between the sex organs of morphs and their fecundity. First, we evaluated the relative growth of sex organs after anthesis. Then, we studied the stigma receptivity along the flower lifespan including its effect on seed production in both morphs. Finally, given the weak reciprocity between the sex organs of morphs of this species, we estimated population genetic diversity parameters in Long- and Short-styled plants to explore differences between them as a result of rates of inbreeding due to different mating strategies. We observed that Long-styled plants and Short-styled plants present different strategies to avoid sexual interference and both of them had negative consequences in the reciprocity between the sex organs of morphs. Long-styled plants exhibited a delay in stigma receptivity and a higher growth rate of the style after anthesis, while Short-styled plants presented higher herkogamy and no delay in stigma receptivity. These findings suggest that the avoidance of self-interference, in stylar dimorphic Narcissus species, seems to be more critical than improving of reciprocity between the sex organs of morphs. This might explain why reciprocal herkogamy (distyly) is rare in the genus.

Keywords: dichogamy; floral morphology; flower development; herkogamy; heterostyly; heterozygosity; Narcissus broussonetii; self-interference; style dimorphism.

Introduction

Flowering plants exhibit an extraordinary diversity of flower forms, many of which are related to variations in the morphology and arrangement of sex organs (Darwin 1877; Barrett 2002). The most predominant sexual condition in angiosperms is hermaphroditism, the presence of both sex organs (pistil and stamens) within the same flower. Hermaphrodite flowers present some advantages, such as resource economy for the plant because traits to attract pollinators serve the dual role
of promoting female and male floral functions (Givnish 1980; Charlesworth and Charlesworth 1987b; Lloyd 1987). However, the proximity of the sex organs may increase the risk of self-pollination with the possible detrimental consequences of pollen discounting (Harder and Wilson 1998) and inbreeding depression (Darwin 1877; Barrett and Glover 1985; Webb and Lloyd 1986). Dichogamy, on the other hand, may have a disadvantage when stigma receptivity does not overlap temporally with pollen presentation among flowers (Lloyd and Webb 1986).

One mechanism with accurate pollen transfer between the sexual organs of different flowers, which simultaneously promotes outcrossing and avoids self-interference, occurs in heterostylous plants and other reciprocal polymorphisms (Barrett 2002). Heterostyly is characterized by the presence of two (diistyly) or three (tristyly) flower morphs with reciprocal positioning in the height of the stigma and the anthers among flowers of different plants within a population (reciprocal herkogamy). Darwin (1877) proposed that the ultimate function of heterostyly is to promote pollination between anthers and stigmas of different morphs, that is cross-fertilization. Lloyd and Webb (1992a, b) extended Darwin’s original hypothesis, proposing an evolutionary model of heterostyly based on phenotypic selection, in which non-reciprocal stylar dimorphism (morphs differ in stigma height but not anther height) was proposed as the intermediate step in the evolution of distyly. Although stylar dimorphism is less frequent than distyly, it occurs in several genera: Lithodora, Clendrea, Anchusa (Boraginaceae), Nivenia (Iridaceae), Linum (Linaceae; Thompson 2005; Ferrero et al. 2009; Sánchez et al. 2010; Ruiz-Martín et al. 2018) and particularly Narcissus, in which the style polymorphism is present in at least 12 species (Barrett et al. 1996; Baker et al. 2000; Barrett and Harder 2005; Pérez-Barrales et al. 2006; Santos-Gally et al. 2013a).

Stylar dimorphic species exhibit two stylar morphs, Long- (L) and Short- (S) styled, where the stamens are in a similar position and differ mainly in the height of the stigma. L-morph plants present the stigma above (approach herkogamy) or, as in Narcissus, at the same height as the anthers, whereas S-morph plants exhibit the stigma well below the anthers (reverse herkogamy) (Fig. 1). Because there is no reciprocal placement of the stigma and the anthers between morphs and there is a high potential for sexual interference in L-morph flowers...
given the lack of anther-stigma separation in Narcissus (Barrett et al. 2000; Arroyo et al. 2002), the presence of some other floral traits that prevent sexual interference within the L-morph is expected (Baker et al. 2000; Pérez et al. 2004; Ferrero et al. 2009; Santos-Gally et al. 2015). Indeed, recent works have shown that L-morph flowers, but not S-morph flowers, of some stylar dimorphic Narcissus species present dichogamy as a mechanism avoiding self-pollination (Cesaro et al. 2004; Simón-Porcar et al. 2015a). These species have incomplete dichogamy and show some overlap between the presentation of pollen and stigma receptivity, only in the L-morph (Cesaro et al. 2004; Simón-Porcar et al. 2015a). The stigma receptivity in most protandrous species is initially delayed by the style growth (Lloyd and Webb 1986; Barrett et al. 1996). However, dynamics of style growth in protandrous style dimorphic species and the influence on reciprocity, and therefore on self-interference, have not been reported.

Narcissus broussonetii presents the weakest reciprocity between sex organs of morphs of any Narcissus species, resulting in fewer opportunities for inter-morph crossings, especially between anthers of the L-morph and stigma of the S-morph, because the distance that separates them is almost twice the distance between the anthers of the S-morph and the stigma of the L-morph or the distance between the anthers and the stigma of the L-morph (Santos-Gally et al. 2015) (Fig. 1). The ultimate consequence of this arrangement could explain the high frequency of L-morph plants, as found in five of six sampled populations where an excess of L-morph plants was reported (Santos-Gally et al. 2015). However, the previous study also showed that the seed set of L-morph plants tended to be lower than that of S-morph plants (Santos-Gally et al. 2015), which seems contradictory with the abundance of this morph in natural populations. Because the stigma of L-morph is positioned well above their anthers, L-morph might present dichogamy, as other style dimorphic Narcissus species, and hand-pollination on stigmas at different developmental stages might explain the previously observed differences in seed set in inter-morph crosses.

In this study we investigated the occurrence of dichogamy and herkogamy in the style dimorphic N. broussonetii and their effect on self-interference avoidance and reciprocity between morphs. First, we estimated the relative growth of sex organs after anthesis and its effect on maturation, and spatial separation and reciprocity between both morphs. Second, we compared the female function of morphs, in terms of the timing of stigma receptivity and effects of flower age on seed production, by carrying out controlled hand-pollinations. Anther dehiscence occurs at flower opening and pollen is exposed to pollinators during the whole flower lifespan, as in other Narcissus species; therefore, we did not evaluate experimentally the male function, which is mostly affected by extrinsic factors (Cesaro et al. 2004; Medrano et al. 2005; Simón-Porcar et al. 2015a). Additionally, given the weak reciprocity between the sex organs of morphs of this species, we estimated the genetic diversity parameters of a natural population of N. broussonetii to explore their possible differences between morphs as a result of different rates of inbreeding in each morph within the population, for example if morphs are spatially and genetically clumped due to low dispersal, leading to mates occurring mostly within morphs (assortative mating; Hodgins and Barrett 2006; Stelhik et al. 2006) or when short-tongued pollinators, which mostly pollinate L-morph flowers, are abundant, favouring assortative mating among L-morph plants (Pérez-Barrales and Arroyo 2010; Santos-Gally et al. 2013b, 2015; Simón-Porcar et al. 2014).

Methods

Study species and population sampling

Narcissus broussonetii is a geophyte endemic of Atlantic central and southern coasts of Morocco (Fernandes 1940), which represents the southernmost range of daffodils. Flowering plants produce up to 15 flowers, which are arranged in an umbel-like inflorescence, from October to early December albeit in a very irregular fashion, in timing and intensity, due to unpredictability of autumn rainfall in this arid sub-Mediterranean region. Narcissus broussonetii is a stylar dimorphic species with an unusual combination of perianth traits among daffodils. It has the longest floral tube of all dimorphic species in the genus (26.9 ± 0.5 mm), where the stamens are adnate in two whorls (three stamens in each one) and a virtual lack of a corona (Fig. 1). Most of the identified populations are anisoplectic where the L-morph predominates (56.1–90.8 %; Santos-Gally et al. 2013a, 2015). It is self-incompatible but morph-compatible, that is crosses between plants of the same morph render similar seed set than crosses between different morphs (Santos-Gally et al. 2015).

Plants used in this study were collected from a natural population in south-western Morocco (29°39′N, 9°26′W, SEV284809) and were transferred to a glasshouse at University of Seville in Spain. We collected plants separated by 1 m or more to avoid repetition of the same genet, as there is some vegetative reproduction. Plants were randomly collected in the population; thus, morph proportion in our sample reflects that in the population and its genetic diversity. We preferred to collect plants from a single population, the largest among those visited, rather than to have a sample collected across smaller populations, to minimize confounding effects of possible among-population differences. Plants were grown in free-draining black plastic pots, containing a standard commercial potting mix (Blumenerde, Gramoflor). Pots were watered until field capacity daily, as it usually occurs in the field during autumnal growing season prior to flowering.

Relative growth of sex organs, herkogamy and reciprocity between sex organs of morphs

We compared dynamics of style growth between morphs using morphometric measurements of flowers from anthesis until Day 6 (day) to determine the differences in the development of sex organs. In this study anthesis is defined as the time when the perianth opens and sexual organs are presented to floral visitors. Floral longevity of Narcissus species present dichogamy as a mechanism to explore their possible inbreeding in each morph within the population, for example if morphs are spatially and genetically clumped due to low dispersal, leading to mates occurring mostly within morphs (assortative mating; Hodgins and Barrett 2006; Stelhik et al. 2006) or when short-tongued pollinators, which mostly pollinate L-morph flowers, are abundant, favouring assortative mating among L-morph plants (Pérez-Barrales and Arroyo 2010; Santos-Gally et al. 2013b, 2015; Simón-Porcar et al. 2014).
traits: floral tube length, style length, upper- and lower-whorl anther height (Santos-Gally et al. 2015) (Fig. 1). We measured only one stamen per whorl. The floral tube was measured as a control for potential allometric relations with the sex organs. Style, upper and lower anther lengths were adjusted using the formula described by Baker et al. (2000), in which: adjusted data = organ position − b (floral tube length − floral tube mean length), where b indicates the slope of the regression of organ position and floral tube length. All measurements were taken from digital images of the side view of flowers using ImageJ (Schneider et al. 2012). For each plant we calculated the total growth of floral tube, style, upper and lower anthers as the difference between the length of each trait when the flower opened and their maximum length after the flower opening and the relative growth for each day. We used generalized estimated equations (function GEE in SPSS 2016, version 24; IBM Corp 2016) to account for allometric effects and morph on sex organ (style, upper anthers or lower anthers) growth, during floral lifespan. Each flower was identified and this information was included in the model as a subject variable. Flower age (1- to 6-day) was defined as a within subject factor, because there were repeated measures of each trait. We included morph as a fixed effect and the floral tube growth as a time-varying covariate. Finally, we selected a gamma distribution with log link and to test model effects type III and Wald chi-square statistic.

Effect of flower age on seed production
We applied six hand-pollination treatments with flowers of different ages (i.e. 1- to 6-day) to determine the effect of flower age on seed production. The hand-pollination treatment was the same as applied above. The position of each recipient flower on the inflorescence of each treatment was randomly selected and the number of replicates per treatment was balanced across flower positions. Plants were grown outside of the glasshouse and each sace and its inflorescence were caged with a mesh (pore size 0.1 mm). Fruits were harvested 6-8 weeks after hand-pollinations and the number of plump seeds, aborted seeds and undeveloped ovules were counted. A total of 50 L- (260 flowers) and 17 S-morph plants (101 flowers) were used in the experiment, making the number of flowers of each treatment similar for each morph. Because the total number of ovules in the ovary is not the same between flowers in different positions in the inflorescence, we estimated the seed set using the formula: seed set = number of seeds developed/total number of ovules in the ovary.

The effect of flower age on seed production was analysed using generalized estimated equations GENLIN for proportion data (function GEE in SPSS 2016, version 24; IBM Corp 2016). A binomial error distribution was used for the dependent variable (number of ovules converted into seeds) and flower age (days), morph and their interaction as categorical predictors. Repeated measurements (plants) and the position of the flower within an inflorescence, which may affect resources available for seed development, were included in the analyses as subject and within-subject variables, respectively. To test model effects, we specified type III sum of squares, chi-square statistic and kernel as a log quasi-likelihood function.

Population genetic diversity parameters
We used 10 microsatellite loci to determine the heterozygosity, inbreeding coefficients and other genetic diversity parameters within the population and between morphs to test for possible genetic differences between morphs. These microsatellite loci were developed for N. papyraceus and have been tested in other Narcissus species (Simón et al. 2010; Barranco et al. 2019). We collected two fresh leaves from each of the plants maintained in the glasshouse (a total of 74 L- and 17 S-plants). Genomic DNA was extracted from silica-dried leaves using the DNeasy Plant Mini Kit (Qiagen Inc., Chatsworth, CA, USA) and quality of DNA was estimated with a Nanodrop 2000 spectrophotometer (Desjardins and Conklin 2010). Polymerase chain reaction was performed in 25 μL of reaction mixture containing 50 ng μL⁻¹ DNA, 1x PCR buffer, 1.5 mM MgCl₂, 0.1 μM forward primer, 0.1 μM reverse primer, 0.2 mM mix dNTPs and 1.25 U Tap polymerase. Samples were incubated in a thermal cycler TC-5000. Thermocycling conditions for the microsatellite loci were 5 min at 94 °C, followed by 45 cycles at 94 °C for 30 s, 50 °C for 30 s (annealing) and 72 °C for 30 s (extension) with a final extension at 72 °C for 5 min. To determine whether amplification was successful we ran 1 μL of the product in an agarose gel. Sequencing was performed using Applied Biosystems Prism model 3700 automated sequencer.
Genetic data analysis

Results were analysed using Peak Scanner v1.0 software and LIZ 500 size standard. Genotyping errors, such as short allele dominance (large allele dropout), stutter peaks and typographic errors were detected with Micro-Checker v 2.2.3 (Van Oosterhout et al. 2004), using 10 000 permutations and 95% confidence interval. For the non-amplified alleles (null alleles), the frequencies were calculated with Null Allele Estimator (for non-equilibrium populations; Van Oosterhout et al. 2006) using the inbreeding coefficient (average for all microsatellite loci) and homozygote frequency observed.

Average number of alleles observed per locus (A) and allelic richness (mean number alleles per locus corrected for the minimum sample size; \(R_o\)) were estimated using FSTAT version 2.3.9 (Goudet 1995). The P-values for the Hardy–Weinberg equilibrium (HWE), inbreeding coefficients \(F_{\text{IT}}\) (mean inbreeding coefficient of subpopulations of L- and S-morph plants, respectively, relative to the total population) and \(F_{\text{is}}\) (mean inbreeding coefficient of L- and S-morph individuals, respectively, relative to the subpopulations of L- and S-morph plants), number of private alleles observed per locus (PA), observed heterozygosity (\(H_o\)) and gene diversity (\(H_e\)) were estimated with GenAIEx v. 6.501 software (Peakall and Smouse 2012). We conducted an ANOVA (in SPSS 2016 version 24) to compare the genetic A, \(R_o\), \(H_o\) and \(H_e\) between morphs, using 10 000 bootstrap replicates and 95% confidence interval. The genetic diversity parameters and inbreeding coefficients were estimated for L- and S-morph plants separately, to compare between them. The genetic structure of L- and S-morph plants of \(N. broussonetii\) was explored using Bayesian analyses with STRUCTURE v2.3.4 (Pritchard et al. 2000; Hubisz et al. 2009). This software assigns individuals (or populations) to clusters (K) using genotype data (unlinked markers). In our analyses, we specified two different clusters (\(K = 2\)), to test whether L- and S-morph plants can be classified into two independent clusters according to their genetic data. We ran the analyses under correlated allele frequencies and admixture models. We specified a burn-in period of 50 000 interactions and 20 000 Markov Chain Monte Carlo (MCMC) repetitions after the burn-in. Finally, we repeated the simulation 10 times for a number of clusters \(K = 2\). Individual’s membership fraction was estimated for all the plants included in the analyses (74 L and 17 S).

Results

Relative growth of sex organs, herkogamy and reciprocity between sex organs of morphs

Upper and lower anther growth was not correlated with floral tube growth nor did it differ between morphs (Table 1).

However, style length was obviously different between morphs, independent of floral tube growth (Table 1). The floral tube grew a total of 1.1 ± 0.2 mm and 0.8 ± 0.3, respectively, for the L- and S-morph, from flower opening until the fourth day after anthesis [see Supporting Information—Fig. S1]. Upper anthers grew a total of 0.9 ± 0.3 mm for the L-morph plants and 0.6 ± 0.5 mm for the S-morph plants and lower anthers grew a total of 1.0 ± 0.4 mm for the L-morph plants and 0.8 ± 0.4 mm for the S-morph plants. Upper and lower anthers grew until the fourth day after anthesis (Fig. 2B). Styles grew a total of 2.4 ± 0.3 mm for the L-morph plants and 0.7 ± 0.5 mm for the S-morph plants. Three days after the flower opened the style grew a total of 1.9 ± 0.3 mm for the L-morph plants and 0.4 ± 0.5 mm for the S-morph plants (Fig. 2A). We also recorded the withering of sex organs after they reached their maximum height (Fig. 2A and B; see Supporting Information—Fig. S1).

The greatest spatial separation between anthers and stigmas was in 2-day flowers for the S-morph (7.9 ± 1.3 mm) and 3-day flowers for the L-morph (6.3 ± 0.7 mm). The lowest spatial separation between anthers and stigmas was in 1-day flowers for the L-morph (5.0 ± 0.5 mm) and 6-day flowers for the S-morph (6.0 ± 1.3 mm). Herkogamy was higher in S- than L-morph flowers for 1- to 5-day flowers (Fig. 2C). Reciprocity was variable across the flower lifespan but L-morph plants always presented stronger reciprocity than S-morph plants. The strongest reciprocity for the L-morph was at flower opening (1.7 ± 0.2 mm) and the weakest was in 3-day flowers (3.8 ± 0.2 mm), when the styles reached their maximum length. The strongest and weakest reciprocity for the S-morph were in 3-day (7.2 ± 0.2 mm) and 6-day flowers (5.9 ± 0.2 mm), respectively (Fig. 3).

Stigma receptivity

The number of germinated pollen grains differed significantly between morphs, flower age and their interaction (Table 2A). The stigma receptivity was high for S-morph flowers throughout the entire observation period (6 days). The stigma receptivity of L-morph flowers was very low during the first 2 days, peaked in the third day and decreased during the fourth to sixth days (Fig. 4).

Effect of flower age on seed production

The number of ovules converted into seeds differed between morphs and flower ages (Table 2B). S-morph plants produced significantly more seeds than L-morph plants. Flowers of both morphs tend to produce most seeds when pollinated during the first day of high stigma receptivity (Day 1 for S-morph flowers, Day 3 for L-morph flowers) and fewest seeds when pollinated in the sixth day of flower lifespan (Fig. 5).

Table 1. Results of the generalized estimated equations analysis for upper and lower anthers and style growth as dependent variable, morph as fixed effect, growth of the floral tube as a covariate and flower age as repeated measures.

<table>
<thead>
<tr>
<th></th>
<th>Sexual organs growth after anthesis</th>
<th>(\chi^2) Wald</th>
<th>P-value</th>
<th>d.f.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper anthers</td>
<td>Morph (L and S)</td>
<td>2.4</td>
<td>&lt;0.115</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Floral tube</td>
<td>0.8</td>
<td>&lt;0.773</td>
<td>1</td>
</tr>
<tr>
<td>Lower anthers</td>
<td>Morph (L and S)</td>
<td>0.1</td>
<td>&lt;0.696</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Floral tube</td>
<td>2.4</td>
<td>&lt;0.115</td>
<td>1</td>
</tr>
<tr>
<td>Style</td>
<td>Morph (L and S)</td>
<td>6.5</td>
<td>&lt;0.010</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Floral tube</td>
<td>0.4</td>
<td>&lt;0.507</td>
<td>1</td>
</tr>
</tbody>
</table>
Population genetic diversity parameters

The Null Allele Estimator analysis showed no presence of null alleles in any locus. The population was not in HWE for any locus and two of the 10 microsatellite loci were monomorphic, A131 and B104 [see Supporting Information—Table S1]. The L- and S-morph plants only showed significant differences between morphs in the average number of alleles and private alleles per locus (Table 3). Allelic richness ($R$), $H_o$ and $H_e$ did not differ between morphs (Table 3). Inbreeding coefficients, $F_{ST}$ and $F_{IS}$, were similar between morphs and close to 0 (Table 3). The L- and S-morph plants were not assigned to independent clusters in the STRUCTURE analysis (Fig. 6). The average individual membership fraction was $0.50 \pm 0.01$ in both clusters for L- and S-morph plants. Posterior probabilities of $K$ were consistent across the 10 repetitions, suggesting that the burn-in and run periods were adequate.

Discussion

Functional implications of herkogamy and dichogamy in Narcissus broussonetii

Our study highlights the importance of considering herkogamy and dichogamy together when studying sex conflicts within the flower of a self-incompatible and morph-compatible style dimorphic species, and their effects on the reciprocity between morphs and reproductive success. Narcissus broussonetii, the daffodil with the southernmost range, presents the highest average herkogamy of all style dimorphic species in the genus (Arroyo and Dafni 1995; Baker et al. 2000; Arroyo et al. 2002; Pérez-Barrales et al. 2006; Santos-Gally et al. 2015). The herkogamy is higher in S- than L-morph flowers (Fig. 2C), probably because approach herkogamy is more efficient at avoiding self-interference than reverse herkogamy, a rare condition in flowering plants (Webb and Lloyd 1986). Moreover, our results showed a gradual increase in the herkogamy for L-morph flowers during the life of the flower, which is principally caused by the higher growth of the L-style after flower opening (Fig. 2A).

Among heterostylous species for which data on style length variation are available, results indicate that styles of L-flowers have longer cells than the styles of S-flowers (Richards and Barrett 1992; Cohen 2016). However, cell size is not sufficient to account for style length variation and thus cell number, and its proliferation must also differ. Indeed, in distylous Rubiaceae two alternatives in the development of the style have been described: a differential relative growth rate or a differential time of growth inhibition (Richards and Barrett 1992; Faivre 2000). According to our results, N. broussonetii exhibits differential relative growth rates of the styles between morphs after flower opening, as styles begin to grow at the same ages for S- and L-morph flowers (Fig. 2A). However, more studies focused in floral ontogeny of style dimorphic Narcissus species will be essential to clarify whether the style dimorphism in this genus is a consequence of a different growth rate of the style. The higher growth rate of the style in L- versus S-flowers after anthesis might be the cause of the delay in the stigma receptivity observed in L-flowers, as it has been described for other protandrous species (Lloyd and Webb 1986; Snow and Grove 1995; Barrett et al. 1996; Langenberger and Davis 2002). The stigmas of L-flowers of N. broussonetii presented a delay in their receptivity of up to 3 days (Fig. 4). However, the stigmas of the S-flowers were receptive at anthesis. The differences in stigma receptivity between morphs were consistent with the patterns of seed production according

Figure 2. Relative accumulated growth (mean ± SE) of sex organs after the anthesis in 1- to 6-day flowers of Narcissus broussonetii. (A) Relative accumulated growth of style. (B) Relative accumulated growth of upper and lower anthers. (C) Herkogamy (mean ± SE) of stigma and upper anther heights for the L-morph and of lower anthers and stigma heights for the S-morph.

Figure 3. Differences between reciprocal sexual organ length (mean ± SE mm) from 1- to 6-day flowers of Narcissus broussonetii. Diamonds represent the differences between the stigma height of the L-morph and upper anthers height of the S-morph and squares the difference between the lower anthers height of the L-morph and stigma height of the S-morph.
interaction (Fig. 4). The seed set (mean ± SE) of L- and S-morphs of Narcissus broussonetii after hand-pollinations on 1- to 6-day flowers. Statistical results are given in Table 2B.

Table 2. Results of the generalized linear model analysis for stigma receptivity (A) and seed set (B) data as dependent variable, morph flower age and their interaction as fixed effects.

<table>
<thead>
<tr>
<th></th>
<th>χ² Wald</th>
<th>P-value</th>
<th>d.f.</th>
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<tbody>
<tr>
<td><strong>A: Stigma receptivity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morph (L and S)</td>
<td>851.1</td>
<td>&lt;0.001</td>
<td>1</td>
</tr>
<tr>
<td>Flower age (1- to 6-day)</td>
<td>360.0</td>
<td>&lt;0.001</td>
<td>5</td>
</tr>
<tr>
<td>Interaction: Morph (L and S) × Flower age (1- to 6-day)</td>
<td>476.7</td>
<td>&lt;0.001</td>
<td>1</td>
</tr>
<tr>
<td><strong>B: Seed set</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morph (L and S)</td>
<td>12.1</td>
<td>0.001</td>
<td>1</td>
</tr>
<tr>
<td>Flower age (1- to 6-day)</td>
<td>24.1</td>
<td>&lt;0.001</td>
<td>5</td>
</tr>
<tr>
<td>Interaction: Morph (L and S) × Flower age (1- to 6-day)</td>
<td>3.8</td>
<td>0.568</td>
<td>5</td>
</tr>
</tbody>
</table>

is no information regarding stigmatic receptivity. Therefore, the occurrence or absence of high herkogamy and dichogamy in Narcissus species might represent a joint strategy along the phylogeny of this genus, as has been described for other floral traits (Santos-Gally et al. 2013a), which might be related with a shift to selfing in monomorphic species or with sex interference avoidance in dimorphic species.

Herkogamy and dichogamy occur combined with self-incompatibility in N. broussonetii. These mechanisms play an important role in helping to avoid the negative consequences of self-interference. This is especially important in species that have late ovarian self-incompatibility with ovule degeneration, as in most self-incompatible Narcissus species (Dulberger 1964; Barrett et al. 1997; Sage et al. 1999; Arroyo et al. 2002; Santos-Gally et al. 2015; Simón-Porcar et al. 2015a). However, the high herkogamy of N. broussonetii determines that this species presents the weakest reciprocity between the sexual organs of morphs of all Narcissus species (Santos-Gally et al. 2015). S-morph plants exhibit weaker reciprocity than L-morph plants, and the reciprocity of this morph is constant across the flower lifespan (Fig. 3). However, the styles of L-morph plants are very close in height to the upper anthers of S-morph plants at the flower opening and after 3 days when the style of L-morph plants reaches its maximum size and is receptive, reciprocity decrease because difference between stigma height of the L-morph plants and upper anther height of the S-morph plants is twice (Fig. 3).

This study showed how S- and L-morph plants of N. broussonetii present different mechanisms to avoid sexual interference. L-morph plants exhibit a steady increment in herkogamy, which produces a delay in the stigma receptivity to avoid sexual interference during the first 2 days after anthesis. This increase in herkogamy is mainly produced by a higher growth of the style, which has negative consequences on the reciprocity between the stigmas and upper anthers of L- and S-morph plants, respectively. S-morph plants present higher herkogamy between their lower anthers and stigma heights, probably to avoid sexual interference within the flowers of this morph. The higher herkogamy of the S-morph also has negative consequences in the reciprocity between its stigma and the lower anthers of the L-morph. In any case, in style dimorphic Narcissus species, avoiding self-interference between sexual functions, seems to be more critical for successful outcrossing than improving morph reciprocity. However, self-interference might be stronger in L-morph flowers, as the stigma is initially closer to the upper stamen whorl and self-pollination might result in high ovule discounting, due to late-acting self-incompatibility (Cesaro et al. 2004). The stigmas of S-morph flowers never pass through the anther levels in the flower; thus, self-interference might be reduced. In contrast, lack of
Table 3. Population genetic parameters (mean ± SD) and inbreeding coefficients ($F_{Is}$ and $F_{St}$) of long- (L) and short- (S) morphs in a population of Narcissus broussonetii. Sample size (N), number of alleles (A), number of private alleles (PA), allelic richness ($R_a$), observed heterozygosity ($H_o$), genetic diversity ($H_e$) and $P$-values of the ANOVA between morphs for all parameters. *Significant differences between morphs ($P < 0.05$).

<table>
<thead>
<tr>
<th>Morph</th>
<th>N</th>
<th>A</th>
<th>PA</th>
<th>$R_a$</th>
<th>$H_o$ (± SE)</th>
<th>$H_e$ (± SE)</th>
<th>$F_{Is}$ (± SE)</th>
<th>$F_{St}$ (± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>74</td>
<td>5.9 ± 1.0*</td>
<td>2.7 ± 0.7*</td>
<td>4.2 ± 0.6</td>
<td>0.61 ± 0.11</td>
<td>0.53 ± 0.09</td>
<td>0.05 ± 0.00</td>
<td>−0.16 ± 0.00</td>
</tr>
<tr>
<td>S</td>
<td>17</td>
<td>3.2 ± 0.4</td>
<td>0</td>
<td>4.2 ± 0.6</td>
<td>0.58 ± 0.10</td>
<td>0.47 ± 0.08</td>
<td>0.05 ± 0.00</td>
<td>−0.22 ± 0.00</td>
</tr>
</tbody>
</table>

In field conditions, the fecundity of both morphs has not been determined. It would be interesting to test if the delay in the stigma receptivity of L-morph plants produces negative consequences in the fecundity of this morph in the field; because the flower is receptive during a shorter period of time (Fig. 4). It is surprising the prevalence of this morph in all sampled populations from Santos-Gally et al. (2015) given this result (Figs 4 and 5). It seems to indicate that L-morph plants of N. broussonetii might transmit more of their genes to the progeny through their pollen than their ovules. This gender specialization could be explored with paternity analysis using microsatellite markers (already available) and it will be the first case of gender specialization observed in Narcissus species, where the prevalence of disassortative mating and no gender differentiation is the norm (Hodgins and Barrett 2006; Simón-Porcar et al. 2015b).

In conclusion, this study demonstrates that anther–stigma reciprocity, a kind of herkogamy involving two sex morphs in a population, is strongly affected by dichogamy. Both mechanisms are thought to decrease sexual interference in flowers, with reciprocal herkogamy suggested as the strongest in promoting the establishment of style dimorphism, aided by the role of active pollinators promoting disassortative effective pollination (Lloyd and Webb 1992b; Simón-Porcar et al. 2015b). Here we propose that dichogamy might alter the outcomes of this mechanism, and might explain in part, together morph-compatibility, the excess of L-morph plants in many style dimorphic species in Narcissus.

Supporting Information

The following additional information is available in the online version of this article—

Table S1. Primer screening in a population of Narcissus broussonetii. Sample size (N), number of alleles ($A$), allelic richness ($R_a$), observed heterozygosity ($H_o$), genetic diversity ($H_e$), inbreeding coefficient ($F_{Is}$) and $P$-values for the Hardy–Weinberg equilibrium test are given for each marker.

Figure S1. Relative accumulated growth (mean ± SE) from 1- to 6-day flowers of floral tube of Narcissus broussonetii.

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Contributions by the Authors

J.A. and R.S.-G. conceived and designed the experiments. D.B. and R.S.-G. performed the experiments. D.B. analysed
the data. All authors contributed to discussions, writing and approved the manuscript.

Conflict of Interest
None declared.

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