

Direct evidence supporting Darwin's hypothesis of cross-pollination promoted by sex organ reciprocity

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Summary

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- The floral phenotype plays a main role in the attraction and fit of pollinators. Both perianth traits and the positioning of sex organs can be subjected to natural selection and determine nonrandom mating patterns in populations. In stilar-polymorphic species, the Darwinian hypothesis predicts increased mating success between individuals with sex organs at equivalent heights (i.e. with higher reciprocity).
- We used paternity analyses in experimental populations of a stilar-dimorphic species. By comparing the observed mating patterns with those expected under random mating, we tested the effects of sex organ reciprocity and perianth traits on mating success. We also analysed phenotypic selection on perianth traits through female and male functions.
- The (dis)similarity of parental perianth traits had no direct effects on the mating patterns. Sex organ reciprocity had a positive effect on mating success. Narrow floral tubes increased this effect in upper sex organs. Perianth traits showed little signs of phenotypic selection. Female and absolute fitness measures resulted in different patterns of phenotypic selection.
- We provide precise empirical evidence of the Darwinian hypothesis about the functioning of stilar polymorphisms, demonstrating that mating patterns are determined by sex organ reciprocity and only those perianth traits which are critical to pollinator fit.

Introduction

'No little discovery of mine ever gave me so much pleasure as the making out the meaning of heterostyled flowers'

(autobiography of Charles Darwin, in Darwin, 1887)

Angiosperm evolution, driven by the interaction of variable floral traits and pollinators, is among the greatest long-term biodiversity drivers on the planet (Darwin, 1862; Grant, 1949; Stebbins, 1970; Friedman, 2009; Johnson, 2010). The floral phenotype plays a main role in the attraction and mechanical fit of pollinators, and natural selection on floral traits optimizes the transfer of gametes and individual fitness (Fenster *et al.*, 2004; Harder & Johnson, 2009; Phillips *et al.*, 2020). How much individuals mate, and with whom, shapes the evolution of floral phenotypes in plant populations.

The floral phenotype, including perianth and sex organ traits, can determine nonrandom mating patterns in populations if the efficiency of pollen transfer is improved within or between particular floral phenotypes due to changes in pollinator's fit and their body's contact areas with sex organs (de Almeida *et al.*, 2013; Newman *et al.*, 2015; Barrett & Harder, 2017; Minnaar *et al.*, 2019), or if different pollinators show constant and divergent phenotypic preferences (Møller, 1995; Schiestl & Schlüter, 2009; de Jager & Ellis, 2012; Kaczorowski *et al.*, 2012).

Nonrandom mating entails either barriers to gene flow between phenotypic or genetic groups, potentially leading to the sympatric differentiation of floral ecotypes (assortative mating; Anderson *et al.*, 2010; see also Rymer *et al.*, 2010), or admixture and maintenance of floral polymorphisms (disassortative mating; Fisher, 1941; Shang *et al.*, 2012). Most plants bear hermaphroditic flowers and have therefore both female and male sex functions, which could lead to conflicting or context-dependent patterns of phenotypic selection and mating (Ashman & Morgan, 2004; Arnqvist & Rowe, 2005; Antunes *et al.*, 2010; Kulbaba & Worley, 2012; Zhou *et al.*, 2020). An analysis of the two sex functions in hermaphroditic plants and their interaction with different pollinator assemblages is therefore essential for a complete understanding of the mechanisms involved in floral evolution.

Stilar polymorphisms, such as heterostyly, exist in populations bearing various floral types that differ in the position of female and male sex organs within the flowers, which are placed at variable but more or less reciprocal positions (Barrett *et al.*, 2000; Armbruster *et al.*, 2017; Barrett, 2019). Darwin (1877) proposed that this reciprocal positioning evolved as a mechanism to promote disassortative mating through efficient pollen transfer between morphs with female and male sex organs contacting different parts of the pollinator's body. Both the deviations from perfect sex organ reciprocity and the diversity of floral visitors (with variable body size, morphologies, fit and pollination efficiencies for each

morph) of most stylar-polymorphic populations affect pollen transfer patterns (Adler & Irwin, 2006; Ferrero *et al.*, 2011; Zhu *et al.*, 2015; Deschepper *et al.*, 2018; Brys & Jacquemyn, 2020). A prerequisite of the Darwinian mechanism is that perianth traits are similar for the two floral morphs and pollinators cannot discriminate between them (Darwin, 1877). However, perianth traits affecting pollinator fit may interact with sex organ reciprocity and affect the pollen transfer efficiency. It has been proposed that narrow floral tubes might boost the efficiency of sex organ reciprocity in disassortative pollination because they restrict pollinator's movements (Lloyd & Webb, 1992a; Santos-Gally *et al.*, 2013).

Heterostylous plants commonly bear heteromorphic self-incompatibility (HetSI) and deviations from disassortative pollen transfer have little impact on mating patterns, leading to equal morph ratios in populations of these species (Barrett & Cruzan, 1994), although high pollen discount may occur. Support for the Darwinian hypothesis in these species relies on studies of pollen transfer under laboratory conditions (e.g. Keller *et al.*, 2014), or on studies in natural populations relating reciprocity, measured at the population level (Sánchez *et al.*, 2013; Armbruster *et al.*, 2017), with female fitness or rates of disassortative pollen transfer measured from stigmatic dimorphic pollen loads (e.g. Jacquemyn *et al.*, 2018; Brys & Jacquemyn, 2020). Unfortunately, in such correlative approaches the real pollination transfer patterns are a black box. In contrast, the mating patterns of stylar-polymorphic plants without HetSI mirror the degree of reciprocity in the position of sex organs and pollen transfer patterns and, as a consequence, morph ratios in natural populations of these plants are variable (Ferrero *et al.*, 2011; Thompson *et al.*, 2012; Ferrero *et al.*, 2017; Matias *et al.*, 2020). These species offer a unique opportunity to test directly the role of sex organ reciprocity in promoting disassortative mating under natural conditions through paternity analyses. However, the studies that have precisely identified mating events in populations at the individual level have analysed mating patterns as a function of reciprocity in a discrete manner (i.e. within or between floral morphs; Hodgins & Barrett, 2008a; Zhou *et al.*, 2015; Simón-Porcar *et al.*, 2015a). An accurate test of the Darwinian mechanism underlying disassortative mating in stylar-polymorphic species depends on measuring sex organ reciprocity in actual mating pairs.

Here we present a quantitative, individual-level approach to analyse the effects of sex organ and perianth traits on the phenotypic selection and mating patterns of *Narcissus papyraceus* under two contrasting pollinator environments. This hermaphroditic, stylar-dimorphic and fully morph-compatible plant species presents a high variation in sex organ reciprocity and perianth traits across its range (Arroyo *et al.*, 2002; Pérez-Barrales *et al.*, 2009). Extensive studies on the natural pollinator assemblages of populations have found that these are dominated by long-tongued insects (butterflies and moths) in the centre and south distribution ranges (long-tongued pollination environment (LTPE hereafter)), and by short-tongued insects (syrphids) in the north range (STPE hereafter; Pérez-Barrales & Arroyo, 2010; Santos-Gally *et al.*, 2013). Short-tongued pollinators, unable to reach the short-styled stigmas, seem to be responsible for the loss of the short-styled morph in northern populations (Arroyo *et al.*, 2002; Santos-Gally *et al.*,

2013; Simón-Porcar *et al.*, 2014). In contrast, perianth traits show random continuous variation within and across populations, independent of style morphs (Pérez-Barrales *et al.*, 2009). This decoupling suggests that sex organ traits are under selection, while perianth traits are not. To test this hypothesis, and to explore the mechanisms of floral evolution in this hermaphroditic plant in depth, we subjected two artificial populations of *N. papyraceus*, built from a pool of 114 parental individuals, phenotyped for eight floral traits and genotyped for four microsatellite markers, to natural pollination under LTPE and STPE. We analysed the effects of floral traits on the mating patterns and on male fitness, estimated from paternity analyses of 760 seedlings, and on female fitness, estimated from seed counts. We investigated Darwin's hypothesis for each pollinator environment by testing whether mating success increased with reciprocity between individuals, explored whether the (dis)similarity of parental perianth traits had effects on the mating patterns of individuals, and tested the role of narrow-tubed flowers in increasing the positive effect of the reciprocity of sex organs on mating success. Finally, we compared the patterns of fitness and phenotypic selection in floral traits through the individual's female and male function.

Materials and Methods

Study species

Narcissus papyraceus Ker-Gawler (Amaryllidaceae) is a winter-flowering geophyte, endemic to the western Mediterranean Basin, with the highest population density and size found at both sides of the Strait of Gibraltar (Arroyo *et al.*, 2002). Individuals present umbels with 3–10 white flowers, bearing a narrow floral tube topped with six free tepals and a wide corona (Fig. 1; Blanchard, 1990; Aedo, 2013), and two whorls of anthers, located inside and on the top of the floral tube, with three stamens in each one. The species bears a high floral phenotypic variation across its range (Pérez-Barrales *et al.*, 2009). There are two style-length morphs: a long-styled morph with the stigma placed at a similar level as the upper anthers, and a short-styled morph with the stigma below the lower anther whorl (Arroyo *et al.*, 2002; Fig. 1). *Narcissus papyraceus* is self-incompatible but fully morph-compatible (i.e. seed set is similar after intramorph and intermorph hand pollinations; Arroyo *et al.*, 2002; Simón-Porcar *et al.*, 2015b). Short-styled plants are abundant in central and southern populations within the LTPE, and absent or very scarce in northern populations within the STPE, where pollinators cannot reach the stigmas of short-styled plants (Arroyo *et al.*, 2002; Pérez-Barrales & Arroyo, 2010; Santos-Gally *et al.*, 2013; Simón-Porcar *et al.*, 2014, 2015a). Although *N. papyraceus* pollinators seem to visit both style-length morphs indistinctly (Simón-Porcar *et al.*, 2014), it is unknown whether they show preferences for particular perianth phenotypes (e.g. Pérez-Barrales *et al.*, 2018).

Experimental setup

In the 2010–2011 flowering season, we exposed two artificial populations of *N. papyraceus* to open pollination in two natural

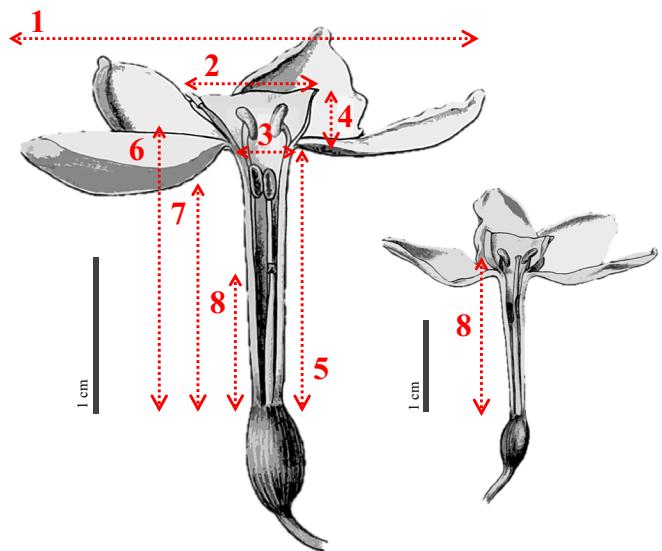


Fig. 1 Short-styled (left) and long-styled (right) flowers of *Narcissus papyraceus* with indication of the floral traits measured in the parental individuals of this study: corolla width (1), corona width (2), tube width (3), corona height (4), tube length (5), upper anthers height (6), lower anthers height (7), style length (8). All floral traits were measured in both morphs.

sites within the LTPE (Finca de la Alcadesa, Cádiz, Spain, 36.3°N, 5.4°W; 64 parental individuals) and STPE (Pinares de Hinojos, Huelva, Spain, 37.3°N, 6.4°W; 50 individuals; Simón-Porcar *et al.*, 2015a). All parental individuals came from a single, big, and phenotypically variable population in the centre of the species range (Playa de Bolonia, Cádiz, Spain; 36.1°N, 5.73°W). We grouped individuals at each site in patches of 8–12 individuals of the two style-length morphs to ensure synchronous flowering among them and to increase the percentage of assignment in the paternity analyses. All subsequent analyses standardized and pooled the data from different patches at each site (see later). This experimental setup maximized our statistical power while resembling real natural populations, where the number of pollen donors fertilizing each maternal individual is limited by phenological mismatches, neighbourhood among individuals, and pollinator behaviour, regardless of the total number of potentially available paternal parents (Weis, 2005; Pannell & Labouche, 2013; Ison *et al.*, 2014; Weis *et al.*, 2014; Ismail & Kokko, 2020).

After flowering, during fruit maturation, we counted the total number of flowers, fruits and seeds produced by each parental individual in order to obtain data on their female fitness. Mating patterns and male fitness were estimated after performing microsatellite-based paternity analyses on three seeds per fruit, on all fruits produced by each parental individual (760 seeds in total). Germination rates (84% overall) and seedling growth were equal for all crosses within and between style-length morphs (Simón-Porcar *et al.*, 2015a). Excluding selfing (9% of genotyped seedlings), we assigned 592 seedlings (78%) to a single paternal individual with an overall assignment probability of 97.6%. Further details on the experimental setup, genotyping and paternity analyses can be found in Supporting Information Methods S1 and S2, and in Simón-Porcar *et al.* (2015a).

Prior to the establishment of the experimental populations, we collected the first flower of each parental individual and conserved them in individually labelled tubes with a 70% ethanol solution. For the purposes of this study, we retrieved those flowers to measure the following floral traits: corolla width, corona width, tube width, corona height, tube length, upper anthers whorl height, lower anthers whorl height and style length (Fig. 1). Measurements were made over scaled photographs of the conserved flowers taken with a Nikon Coolpix 4500 digital camera and analysed with the software IMAGEJ (Rasband, 2012). Corona width, tube width and corona height were estimated as the average values of two measures (maximum and minimum); anthers whorl heights were estimated as the average values of the three anthers in each whorl, measured at the filament insertion level. Across all individuals, all floral traits followed a normal distribution, except for the style length which was bimodal, as expected from its style-length dimorphism (Arroyo *et al.*, 2002). It was not possible to measure flowers subjected to pollinators, but measurements in a control group showed strong correlations of floral traits within *N. papyraceus* inflorescences ($r > 0.9$, $df = 66\text{--}69$, $P < 0.001$; Methods S2). Thus, we assumed there were equal floral traits for all flowers in each parental individual.

Effects of perianth traits on mating patterns

We explored the effects of the (dis)similarity of parental perianth phenotypes on the mating patterns detected through the paternity analysis of genotyped seedlings. Because the two style-length morphs did not differ in any of the other seven measured perianth and anther traits (Fig. 1; $F < 1.213$, $df = 81\text{--}82$, $P > 0.27$; Table S1), the data from both morphs were pooled. For each pollination environment, we first calculated the within-mating pair correlations of each perianth trait (corolla width, corona width, tube width, corona height, tube length). To test the significance of these correlations, we generated null hypothesis distributions for each within-mating pair correlation of traits by using a randomization procedure, as many of the traits were not normally distributed within patches. In each patch, we randomly resampled as many pairs of flowers as the number of seeds with paternity assignment, excluding selfing and allowing replacement (i.e. allowing the same flower to participate in more than one pair). We repeated this process 10 000 times per patch, and for each such sample of mating flowers we calculated the Pearson correlation coefficient between maternal and paternal trait pairs, so as to obtain their distributions under the null hypothesis of random mating. In general, null distributions had a negative mean because of the excluded selfing (Fig. S1). Then, we pooled the null Pearson distributions of different patches at each site and calculated the P -value as the proportion of randomizations that generated a correlation which was equal or higher (in absolute value) than the correlation obtained from the original pairings. We considered a greater or lower correlation than expected under random mating as indicative of assortative or disassortative mating, respectively. Two patches in the STPE were excluded from these analyses, because floral traits could only be measured in two parental individuals from each of these patches.

Effects of sex organ reciprocity on mating patterns

We investigated the effects of the reciprocity of sex organs on the mating patterns by calculating the mismatch (difference) between the heights of the maternal stigma and paternal anthers in each mating pair. The analyses were performed separately for the upper and lower sex organ levels. For each pollination environment, we calculated the mismatch for each mating pair detected in the paternity analysis to generate an observed mismatch distribution, and we tested whether it was significantly different from what was expected under random mating. To generate the null hypothesis distribution for each site, we simulated the pairing for every possible combination of mating individuals within each patch, excluding selfing and considering the number of flowers in each individual to determine the number of possible mating events. We calculated the mismatch for each simulated mating pair and pooled the values for each site. Given their unevenness, we used the nonparametric Wilcoxon rank-sum test to contrast the observed and the null mismatch distributions. Following the Darwinian hypothesis, we expected the observed mismatch to be lower than the one expected under random mating.

Interactive effects of sex organ reciprocity, tube width and pollinator environment on mating patterns

The efficiency of sex organ reciprocity in disassortative pollination may depend on narrow floral tubes restricting pollinator's movements, which may also depend on the type of pollinator. We complemented our previous analysis by exploring which factors (mismatch, tube width, pollinator environment and their interactions) explained the mating patterns in our experimental populations. For that, we built a data frame with every possible combination of mating individuals in our experimental populations and the number of produced seeds (assigned in the paternity analysis, range 0–18), including mismatch, tube width, pollinator environment, the number of possible matching flower pairs, and the spatial distance between individuals. We scored tube width for each mating combination as the sum of maternal and paternal tube widths because we expect the phenotype of both parents to have a similar effect. The number of possible matching flower pairs, which we expected to influence mating probability for each individual pair, was calculated as the product of maternal and paternal number of flowers. The spatial distance between individuals was calculated with the use of trigonometry from their known positions within each circular patch (2 m in diameter). We defined a full generalized linear model (GLM), for each sex organ level mismatch, as:

$$\text{Seeds} \sim \text{Mismatch} \times \text{Tube width} \times \text{Pollinator environment} \\ + \text{Matching flower pairs} + \text{Spatial distance},$$

using a negative binomial distribution to account for the overdispersion and zero excess in the response variable. We used a model selection approach based on Akaike's information criterion with small sample correction (Second-order Akaike Information

Criterion) as implemented in the MuMIN R package (Barton, 2021) to find the explanatory variables that best explained our data. The best-fitting model and those with $\Delta\text{AIC}_c < 2.0$ were considered competitive in explanatory power and were used to calculate the full-average model and the relative importance of the explanatory variables included (Burnham & Anderson, 2002).

Reproductive success

To obtain standardized measures of fitness across experimental patches and sexes, we calculated female, male and absolute reproductive success of each parental individual relative to their patch (Elle & Meagher, 2000; Hodgins & Barrett, 2008b). Female reproductive success (λ_F) was calculated as the number of seeds produced by the individual divided by the total number of seeds produced in the respective patch. Male reproductive success (λ_M) was calculated as the number of seeds sired by the individual divided by the total number of seeds with paternal assignment in the respective patch. The absolute reproductive success (λ_A) was calculated as the average of λ_F and λ_M . All three measures of reproductive success followed a normal distribution.

To explore the relationship between the female and male components of reproductive success in hermaphroditic individuals, and to test whether λ_F and λ_M were good predictors of λ_A , we used Pearson's correlations between each pair of measures in the whole dataset and subsets of data for each pollination environment and morph. We also explored the compliance of Bateman's prediction of a greater variance of reproductive success in male than female function (Bateman, 1948; Christopher *et al.*, 2020) by comparing the variance of λ_F and λ_M with a Levene's test of homogeneity of variance for the same data sets. Finally, we investigated the variation in individual fitness with a two-way ANOVA for the effects of pollination environment and morph on λ_A . We did not analyse λ_F or λ_M here, because prior analyses did not find differences between pollination environments and morphs (Simón-Porcar *et al.*, 2015a).

Phenotypic selection

The flower phenotypic data from both morphs were pooled to analyse phenotypic selection. For the seven perianth and anthers traits, we first explored their associations with principal components analysis (PCA) and Pearson's correlation. The first two principal component axes explained only moderate variance in the dataset (62%; Fig. S2) and were not used to reduce the dimensionality of our dataset. However, we found strong correlations between the floral tube length and the height of the two anther whorls, as expected for epipetalous stamens (Table S2). Therefore, we excluded the height of both anther whorls from the analyses of phenotypic selection, which finally included corolla, corona and floral tube widths, corona height and tube length. The number of flowers, counted at the end of the experiment, was also included in these analyses. Independent tests of phenotypic selection on style length were also performed for each morph (see later).

We carried out analyses of phenotypic selection with female, male and absolute reproductive successes as measures of fitness for the whole experiment dataset by following the standard procedure of Lande & Arnold (1983). We first calculated selection differentials by using univariate regressions between either λ_F , λ_M or λ_A and each standardized floral trait (mean 0, SD 1). A Bonferroni correction of P -values was applied to detect significant selection differentials (corrected error rate P -value = 0.008). To reduce the confounding effects of indirect selection, we also estimated selection gradients by using multivariate regression analyses (Lande & Arnold, 1983). All standardized floral traits, as well as their respective quadratic components, were included as predictor variables in the models with either λ_F , λ_M or λ_A as response variable. All traits in each model had variance inflation factors (VIFs) < 1.54. We report the stabilizing/disruptive selection differentials, which are the double of the quadratic regression coefficients (Stinchcombe *et al.*, 2008). We compared the patterns of phenotypic selection between the two pollination environments and morphs by using analyses of covariance (ANCOVAs) analogous to the selection differentials and selection gradients. We included the corresponding standardized floral traits, pollination environment, morph and the two-way interactions of pollination environment and morph with each floral trait as predictors in the models, with either λ_F , λ_M or λ_A as a response variable. Phenotypic selection on style length was analysed for each morph separately, with univariate regressions and ANCOVAs comparing selection differentials between the two pollination environments.

To compare the patterns of phenotypic selection between female and male functions, we pooled λ_F and λ_M values and used further analogous ANCOVAs to analyse λ as a function of λ type, the corresponding standardized floral traits, and the two-way interactions of λ type with each floral trait as predictors in the models. This procedure was repeated to detect differences between λ_F and λ_A , and between λ_M and λ_A . All analyses were performed with R (R Core Team, 2019).

Results

Effects of perianth traits on mating patterns

There were no significant within-pair correlations for any tested perianth traits (corolla width, corona width, tube width, corona height and tube length) at either pollination environment (Fig. 2), meaning that individuals mated randomly with regard to their perianth phenotype. Corona height presented nearly significant assortative mating in the STPE ($r = 0.255$; $P = 0.053$; Fig. 2).

Effects of sex organ reciprocity on mating patterns

The observed mismatch between the maternal and paternal sex organs of mating individuals differed from expectations under random mating, indicating a greater mating success for more reciprocal individuals (Fig. 3). There was a significantly lower mismatch than expected between short styles and lower anthers in the LTPE ($P = 0.001$), and between long styles and upper anthers in the STPE ($P = 0.001$).

Interactive effects of sex organ reciprocity, tube width and pollinator environment on mating patterns

Seven models presented the lowest AICc values ($\Delta\text{AICc} < 2.0$) for the upper, and six models for the lower sex organs level (Table S3). The averaged best-fitting models for both levels of sex organs presented similarities (Table 1). (1) Following the Darwinian hypothesis, mismatch had a negative effect on mating success (number of assigned seeds); (2) floral tube width also had a negative effect, indicating that narrow floral tubes increase mating success; (3) the number of flower pairs had a positive effect; (4) the spatial distance between individuals had a negative effect; and (5) the number of assigned seeds was lower in the STPE, as expected from the lower number of experimental individuals at this site. In addition, both models included the interaction of floral tube width with pollinator environment, although with opposite effects (Table 1). The negative effect on mating success in the model for upper sex organs indicated that narrow floral tubes increased the mating success in the long-styled morph, especially in the LTPE. The positive effect on mating success in the model for lower sex organs indicated that narrow floral tubes increased mating success in the short-styled morph, especially in the STPE. Finally, the model for upper sex organs also included a negative effect for the interaction of mismatch and floral tube, and a positive effect for the interaction of mismatch and pollinator environment. These effects indicate that the positive effects of reciprocity in mating were stronger for narrow-tubed parents and in the STPE (Table 1).

Reproductive success

Female (λ_F) and male (λ_M) reproductive successes of individuals were uncorrelated ($r < 0.258$; $P > 0.246$; Table S4), but both were good predictors of the absolute reproductive success (λ_A). In contrast to Bateman's prediction, λ_F showed greater variation than λ_M ($F_{1,98-222} > 3.618$; $P < 0.059$). This provoked a greater correlation between λ_F and λ_A ($r > 0.754$; $P < 0.001$) than between λ_M and λ_A ($r > 0.572$; $P < 0.003$). The absolute reproductive success of individuals did not vary between pollination environments or morphs ($F_{1,108} < 0.588$; $P > 0.445$).

Phenotypic selection

Suggesting lack of selection, most perianth traits were uncorrelated with individual fitness, measured as either λ_F , λ_M or λ_A , in the univariate ($t < 2.576$; $P > 0.012$; significance threshold = 0.008) and the multivariate ($t < 1.966$; $P > 0.053$) models (Fig. 4; Table S5). There were differences in the significant results detected in λ_F and λ_A models: corolla width showed a significant positive linear selection coefficient through λ_F ($t = 3.369$; $P = 0.001$), and tube length showed positive and stabilizing selection in the λ_M and λ_A selection gradients ($t > 2.269$; $P < 0.027$). Phenotypic selection patterns were similar for both pollination environments and morphs (interaction terms in univariate ANCOVAs: $F_{1,74-108} < 3.806$; $P > 0.055$; in multivariate ANCOVAs: $F_{1,59-60} < 3.871$; $P > 0.054$; Table S6). Style

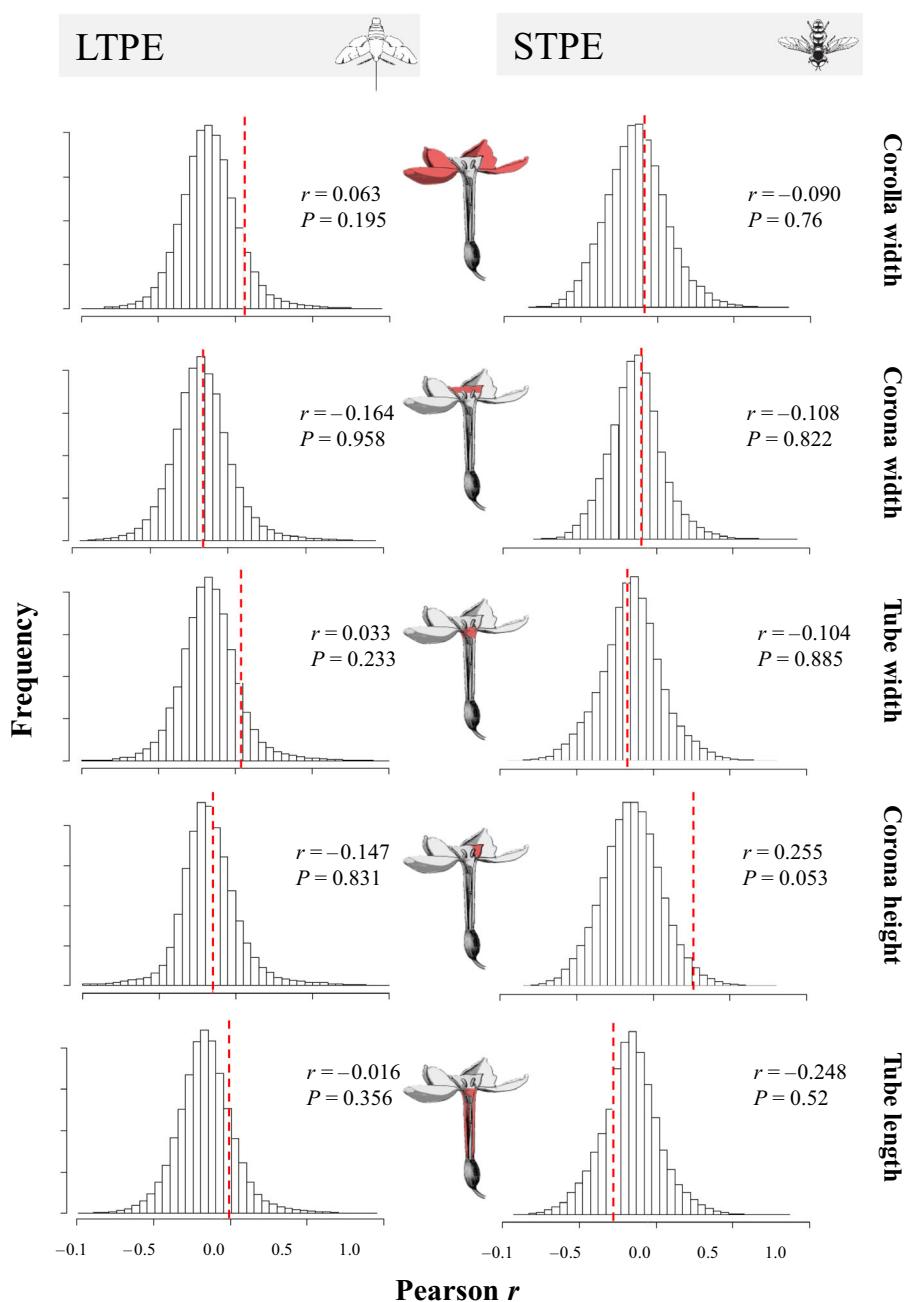


Fig. 2 Within-pair correlations of perianth traits of *Narcissus papyraceus* in each pollination environment, including null distributions (open bars) under the hypothesis of random mating and observed values (red dashed lines) of the Pearson correlation coefficient. LTPE, long-tongued pollination environment; STPE, short-tongued pollination environment.

length was not under selection for any morphs and with any fitness measures ($t < 1.683$; $P > 0.1$), and there were no differences between the pollination environments (interaction terms in univariate ANCOVAs: $F_{1,35-39} < 2.568$; $P > 0.117$; Table S7).

The ANCOVAs for sex function did not detect significant differences in phenotypic selection patterns between λ_F and λ_M , either between λ_F and λ_A , or between λ_M and λ_A , for most floral traits (interaction terms with λ type in univariate ANCOVAs: $F_{1,156-222} < 2.779$; $P > 0.097$; in multivariate ANCOVAs: $F_{1,146} < 2.713$; $P > 0.102$; Table S8). The selection in corolla width (one of the two traits under selection in the former analyses) differed between λ_F and λ_M , with negative estimates for λ_M

(interaction term in univariate ANCOVA $F_{1,161} = 6.607$; $P = 0.011$; in multivariate ANCOVA: $F_{1,147} = 6.551$; $P = 0.011$).

Discussion

Mating patterns

The mating patterns of *N. papyraceus* were independent of the (dis)similarity of parental perianth traits in our experimental populations. This result indicates that neither the LTPE nor the STPE pollinators had phenotypic preferences, a conclusion in line with their generalist nature (Arroyo *et al.*, 2002; Pérez-Barrales *et al.*, 2007; Santos-Gally *et al.*, 2013). It also indicates

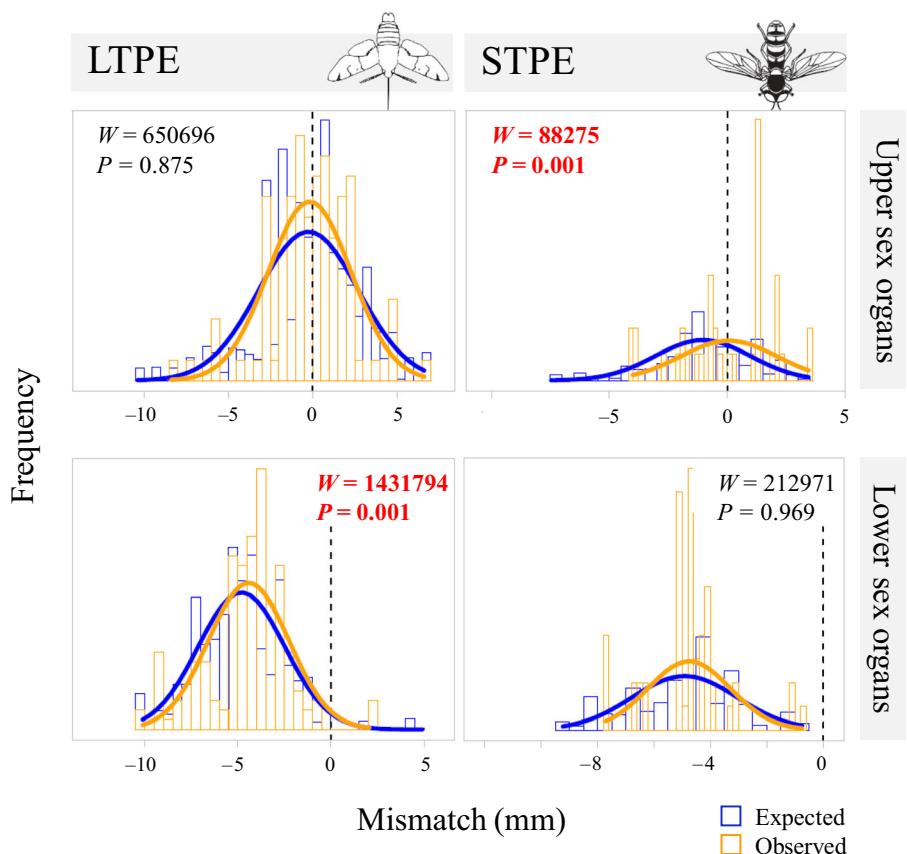


Fig. 3 Distributions of the observed and expected mismatch values of maternal stigma and paternal anthers under random mating, for each sex organs level, in the experimental populations of *Narcissus papyraceus* at each pollination environment. The results of Wilcoxon rank-sum test comparing observed vs expected values are provided in each plot. A normal probability curve has been fitted to the histograms to ease visualization. LTPE, long-tongued pollination environment; STPE, short-tongued pollination environment.

that their pollination efficiency did not vary with perianth traits. Nevertheless, the nearly significant signs of assortative mating for corona height in the STPE might be biologically meaningful. The corona height might be a fit trait by influencing the access of short-tongued pollinators to the upper sex organs, and could determine variable contact areas between them. Detailed observations of short-tongued pollinator visits and insect captures may confirm this hypothesis, which would expand the already known role of corona in *N. papyraceus* pollination (Pérez-Barrales *et al.*, 2018). Together with the perianth similarity of both style-length morphs, the lack of significant effects of perianth traits on mating patterns discards any possible masking effects and fulfils the prerequisites for the accurate functioning of sexual reciprocity in promotion of disassortative mating.

Our two independent analyses support the Darwinian hypothesis (Darwin, 1877; Lloyd & Webb, 1992a,b) by showing that the reciprocal positioning of maternal and paternal sex organs influences mating patterns in both pollination environments. First, we compared the observed and expected mismatch distributions and found that more reciprocal individuals at the upper-level sex organs had greater mating success at the STPE. Second, more reciprocal individuals at the lower-level sex organs had greater mating success at the LTPE. These results perfectly mirror the fit of the two different pollinator types: short-tongued pollinators can only access upper-level sex organs, while long-tongued pollinators can access lower sex organs, and do not necessarily contact upper sex organs when feeding nectar (Fig. S3). The

results of the model selection approach were congruent with this interpretation. The averaged best-fitting models for both levels of sex organs presented a negative effect of mismatch on mating success, and the upper sex organs model indicated that the negative effect of mismatch in mating was stronger in the STPE. The evolutionary consequences of this Darwinian mechanism for the functioning of stylar polymorphisms should proceed through a positive selection of individuals with higher reciprocity (adaptive accuracy; Armbruster *et al.*, 2017) in both monomorphic populations in STPE and dimorphic populations in LTPE. In theory, this might also lead to the maintenance of the polymorphism through disassortative mating between morphs in dimorphic populations (Lloyd & Webb, 1992a,b), and, in the long run, to the transition from stylar dimorphism to reciprocal herkogamy (Darwin, 1877; Ferrero *et al.*, 2011; Simón-Porcar, 2018). Remarkably, although our parental individuals had anthers at similar positions in both morphs, studies in various dimorphic natural populations of *N. papyraceus* have shown that lower anthers are at lower positions in long-styled than in short-styled plants, increasing the reciprocity between the two morphs (Pérez-Barrales & Arroyo, 2010). Future research linking individual reciprocity measures with *ad hoc* data on pollinator visits, as well as extensive studies correlating the reciprocity of morphs with morph ratios in *N. papyraceus* populations, will provide additional support for the hypotheses tested here.

Our results contrast with the conclusions of prior studies which assessed the pollination (Simón-Porcar *et al.*, 2014) and

Table 1 Estimates and standard errors for the averaged best fitting ($\Delta AIC_c < 2.0$) negative binomial generalized linear models (GLMs) modelling mating success of *Narcissus papyraceus* as a function of sex organ mismatch, parental tube widths, pollinator environment (PE; STPE: short-tongued pollination environment), number of possible matching flower pairs and spatial distance between individuals, for upper and lower sex organs.

	Estimate	SE
Upper sex organs		
Intercept	1.449	1.581
Flowers	0.066	0.014
Distance	-0.002	0.002
Mismatch	-0.102	0.278
PE (STPE)	-0.674	1.123
Tube	-0.552	0.283
PE (STPE): tube	-0.373	0.572
Mismatch: tube	-0.052	0.147
Mismatch: PE (STPE)	0.198	0.163
Lower sex organs		
Intercept	-0.675	1.093
Flowers	0.060	0.014
Distance	-0.007	0.002
Mismatch	-0.043	0.07
PE (STPE)	-3.269	3.992
Tube	-0.023	0.321
PE (STPE): tube	1.405	0.735

mating (Simón-Porcar *et al.*, 2015a) patterns of *N. papyraceus* morphs. These previous studies determined that efficient pollination had been driven only by long-tongued pollinators. Indeed, theoretical expectations (Lloyd & Webb, 1992b) and a large body of literature on distylous species (e.g. Alves dos Santos, 2002; Yuan *et al.*, 2017; Abdusalam *et al.*, 2022) have generally assumed that because of the fact that only long-tongued

pollinators are able to reach the low level sex organs and transfer pollen disassortatively between the two morphs, they are the only ones who can transfer pollen precisely. However, our results indicate that the reciprocal positioning of sex organs intrinsically influences pollination efficiency, regardless of the pollinator type involved. They also stress the critical need to quantify the reciprocity and test its role in mating success at the individual level in order to draw correct conclusions about the functioning of stylar polymorphisms.

The evolution of reciprocal stylar polymorphisms has been associated, with exceptions, to narrow-tubed flowers, which constrain pollinator movements within the flower and thus favour precise pollen deposition and transfer (Lloyd & Webb, 1992a; Barrett & Shore, 2008; Santos-Gally *et al.*, 2013; see also Simón-Porcar, 2018). Our empirical results support this hypothesis for the first time, at least partially. The best fitting model of mating success for upper sex organs indicated that the positive effects of reciprocity in mating were stronger for narrow-tubed parental individuals, although such effects were absent in the lower sex organs model. Floral tube width alone, and its interaction with pollinator environment, also influenced the mating success. Narrow floral tubes increased mating success in both levels of sex organs, which is congruent with a closer fit of insects with sex organs (Campbell *et al.*, 1997; Kulbaba & Worley, 2012). This effect was stronger for the long-styled morph in the LTPE and for the short-styled morph in the STPE, suggesting a more important role of narrow floral tubes in less efficient pollinator environments. Nonetheless, an absent or negative correlation of our tube width measurement at the level of upper sex organs with the tube width at the low level might have obscured the interaction of tube width and lower sex organs in our analyses. Studies with more precise measurements of tube width should explore these hypotheses in the future.

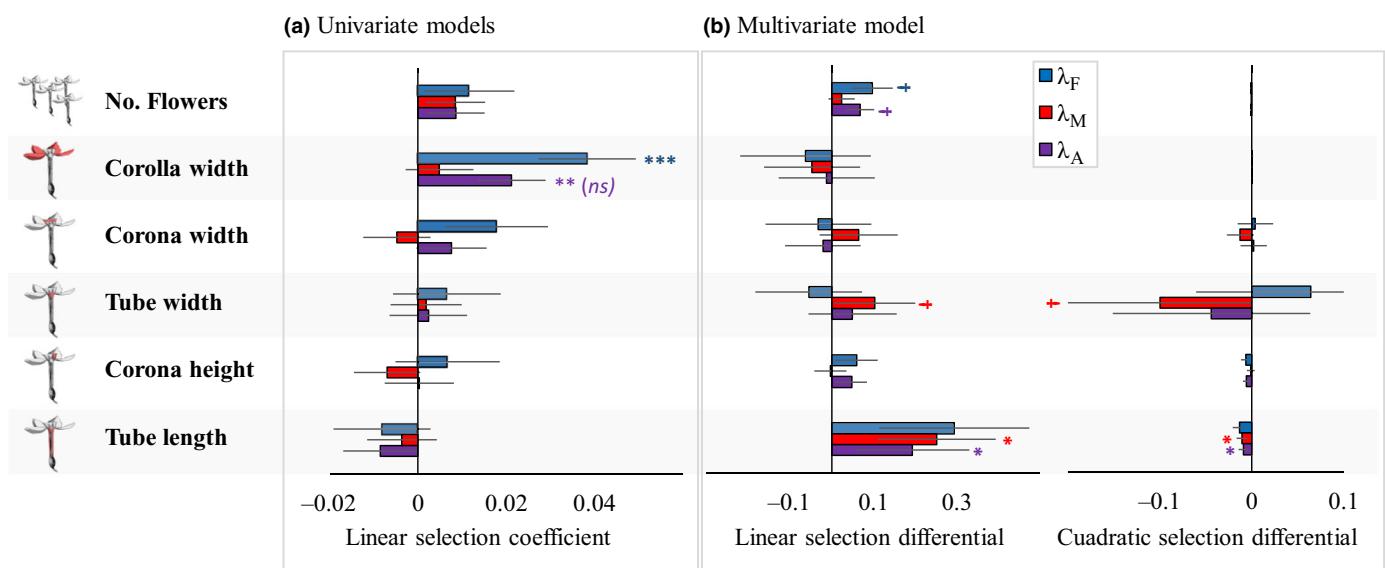


Fig. 4 Phenotypic selection on floral traits of *Narcissus papyraceus* calculated in experimental populations. Estimates ($\pm SE$) from (a) univariate and (b) multivariate regressions of female (λ_F), male (λ_M) and absolute (λ_A) reproductive success of individuals on floral traits. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; †, $P < 0.1$. (ns) not significant after Bonferroni correction of P -values in multiple univariate models.

Reproductive success and phenotypic selection

The random variation in perianth traits among individuals, morphs, natural populations, and pollination environments of *N. papyraceus* (Pérez-Barrales *et al.*, 2007, 2009; but see Pérez-Barrales *et al.*, 2014) seems to respond to the lack of selection in most traits, for both morphs and for both pollination environments. This contrast with the results of sex organ reciprocity on mating patterns, and with the geographic distribution of style-length morphs in the species (Pérez-Barrales *et al.*, 2007; Santos-Gally *et al.*, 2013), suggests that perianth traits are less susceptible to the effects of the divergent pollination environments than sex traits. The strong effects of sex organ positioning on the mating success might hamper any other major selective forces on the floral phenotype of *N. papyraceus*. Furthermore, our results contrast with the idea that the loose fit of short-tongued pollinators with flowers is unlikely to impose selective pressures on floral traits (Stebbins, 1970; Poblete Palacios *et al.*, 2019).

In our experimental populations, only corolla width showed positive linear selection in the univariate λ_F model, which could respond to positive effects on the attraction of pollinators (e.g. Conner & Rush, 1996; Parachnowitsch & Kessler, 2010; Kariyat *et al.*, 2021). Tube length, a trait that has been related to pollen delivery (Muchhal & Thomson, 2009), showed positive and stabilizing selection in the λ_M and λ_A selection gradients. These results support the view that fine phenotypic selection on floral traits is uncommon in Mediterranean ecosystems (Herrera, 1996), and mostly integrative in *Narcissus* (Pérez-Barrales & Arroyo, 2010; Pérez-Barrales *et al.*, 2014).

The use of female fitness as surrogate of absolute fitness in hermaphroditic plants is widespread in floral evolution research (e.g. Bigio *et al.*, 2017; Soteras *et al.*, 2020). However, the few studies estimating the male fitness component in hermaphroditic plants through genetic paternity analyses provide mixed results about its correlation with female fitness and, sometimes, reveal different patterns of phenotypic selection for each sex function (Broyles & Wyatt, 1990; Sahli & Conner, 2011; Briscoe Runquist *et al.*, 2017; Zhou *et al.*, 2020). Here, we found that female (λ_F) and male (λ_M) fitness of individuals were uncorrelated, although both, especially λ_F , were good predictors of the absolute fitness (λ_A). However, this correlation did not translate to similar phenotypic selection patterns for λ_F and λ_A models. All significant selection coefficients and differentials differed between the λ_F and λ_A models, and the ANCOVAs including sex function detected different selection regimes on corolla width (one of two traits showing selection) between λ_F and λ_M . Taken together, our results cannot rule out the existence of conflicting patterns of selection through female and male functions in *N. papyraceus*, and further emphasize the need to analyse male fitness to fully understand phenotypic selection patterns in hermaphroditic plants.

Conclusions

For the first time, our study has quantitatively approached the role of floral traits on the among-individual mating patterns of individual hermaphroditic plants. By appraising female and male

components of fitness through paternity analyses, we have proven that the reciprocal position of sex organs, rather than floral perianth traits, determine the mating patterns in *N. papyraceus*, which supports the Darwin (1877) and Lloyd & Webb (1992a,b) hypotheses for the mechanism of stylar polymorphisms promoting outcrossing through disassortative mating. In contrast to selection of sex organ reciprocity, pollinators of this species are generalist and did not impose major selective pressures of perianth traits related to flower attractiveness. Results show clearly that pollinator mediated selection on flowers may play different roles on different functional traits.

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Author contributions

VIS-P and JA designed the research, VIS-P and AdC conducted field and laboratory work, VIS-P and AJM-P analysed the data, VIS-P wrote the manuscript with contributions of AJM-P and JA.

Competing interests

None declared.

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Data availability

The data that support the findings of this study are available at <https://zenodo.org> with the doi: 10.5281/zenodo.6542400.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Negative within-pair correlation of any given trait when excluding selfing.

Fig. S2 Variance explained by principal components of floral traits of *Narcissus papyraceus*.

Fig. S3 Graphical representation of the significant results of mismatch for each level of sex organs and pollination environment in experimental populations of *Narcissus papyraceus*.

Methods S1 Methods flow chart for the analysis of mating patterns and phenotypic selection in experimental populations of *Narcissus papyraceus*.

Methods S2 Experimental setup, molecular procedures and paternity analyses in *Narcissus papyraceus*.

Table S1 Differences in floral traits of *Narcissus papyraceus* between floral morphs and pollination environments.

Table S2 Pearson correlations between floral traits of *Narcissus papyraceus*.

Table S3 Best fitting models for observed mating success of *Narcissus papyraceus* individual pairs as a function of number of flowers, mismatch, pollinator environment (PE), floral tube width and their interactions, for each level of sex organs.

Table S4 Pearson correlations and results of Levene's test of homogeneity for the female, male and absolute fitness of *Narcissus papyraceus* individuals.

Table S5 Selection differentials and selection gradients resulted from the phenotypic selection analyses on the seven measured floral traits of *Narcissus papyraceus*.

Table S6 ANCOVAs regressing *Narcissus papyraceus* individual fitness on each or all floral traits and their interactions with floral morph and pollination environment.

Table S7 Selection differentials and ANCOVAs regressing style length with individual fitness (and its interaction with pollination environment) for each floral morph (L, S) in *Narcissus papyraceus*.

Table S8 ANCOVAs regressing *Narcissus papyraceus* individual fitness on each or all floral traits and their interactions with sex function (female, male and absolute fitness).

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