

RESEARCH PAPER

The function of the floral corona in the pollination of a Mediterranean style dimorphic daffodil

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Diurnal and nocturnal pollination; flower corona; flower fragrance; flower reflectance; moths; style polymorphism; syrphids; winter flowering.

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ABSTRACT

- *Narcissus papyraceus* is a style dimorphic species with two floral forms, with anthers at similar height and stigmas above (long-styled L) and below (short-styled S) the anther level. The species is self-incompatible, but intra- and inter-morph compatible. Populations are either dimorphic (including both morphs) in the region of the Strait of Gibraltar, or L-monomorphic (with only L plants) in the inland of the Iberian Peninsula. This variation correlates with the most common floral visitors, being primarily long-tongued and short-tongued pollinators, respectively, a rare condition in Mediterranean plants. The maintenance of S-flowers relies on long-tongued insects, as only those deliver pollen to short-styled stigmas. *Narcissus* flowers present a long and narrow tube, at the bottom of which nectar accumulates, and a floral corona, which has been proposed as an important trait for the attraction of pollinators. Here we tested the importance of the corona on pollination of L and S flowers.
- We described UV reflectance patterns of the corona and tepals, and characterised VOCs in intact flowers and flowers with trimmed coronas. We also conducted a field experiment in the dimorphic and monomorphic region to estimate the importance of corona removal on seed production in stands with solitary plants and in groups to control for compatible pollen limitation.
- Reflectance was higher in the tepals than the corona, although both traits presented a reflectance peak around 450 nm wavelength. L- and S-flowers produced similar volatiles, regardless of the manipulation of the corona. Across dimorphic and monomorphic regions, S-flowers with the corona removed suffered a reduction in seed production of ca. 50%, while seed production remained similar in L flowers both with the corona intact and removed. Plants in solitary stands suffered a strong reduction in seed production, which was more pronounced in the monomorphic region.
- Our results suggest that the corona in *Narcissus* is more important for the pollination of S-flowers, which generally have lower seed production compared to L-flowers. Taken together, these results suggest that the floral corona indirectly plays an important role for maintenance of the polymorphism.

INTRODUCTION

Sprengel (1793) first proposed the hypothesis of pollinators as ecologically important for plant reproduction. His work inspired Darwin to interpret pollination interactions with an evolutionary perspective (reviewed in Barrett 2010), when he recognised cross-pollination and avoidance of selfing as important mechanisms to increase female fitness, and pollinators as relatively efficient agents for pollen pick up from anthers and deposition to stigmas (Darwin 1876, 1877). However, the vast majority of angiosperms are hermaphrodite, and frequently pollen lands on the stigma of the same flower. Flowering plants have evolved mechanisms to avoid self-pollination and promote cross-pollination, such as spatial (herkogamy) and temporal (dichogamy) separation of sex organs within a single

flower (Lloyd & Webb 1986; Webb & Lloyd 1986) and genetic incompatibility systems (de Nettancourt 1997), but the counterpart of these mechanisms is the increasing dependency of plants on animal pollinators. Thus, plants have evolved complex mechanisms to attract and facilitate the role of pollinators. Visual floral traits and chemical (olfactory) stimuli are particularly relevant for attraction (Borges *et al.* 2003; Armbruster *et al.* 2005; Raguso 2008; Schiestl 2015), whereas perianth traits and the position of sex organs facilitate the function of pollination (Armbruster *et al.* 2009).

Stylar polymorphisms, including style dimorphism, heterostyly and related cases (Barrett 2002), are among the most studied floral adaptations in the context of avoidance of selfing and promotion of efficient cross-pollination. Style dimorphic species present two floral morphs, with anthers placed virtually at

the same height but with different position of stigmas (e.g. flowers present the stigma either above or below anther level), whereas heterostylous species include two (sometimes three) discrete floral morphs with reciprocal placement of anthers and stigmas (Barrett *et al.* 2000). Lloyd & Webb (1992) proposed that stylar polymorphisms are likely to evolve if appropriate pollinators place pollen from the different morphs on different parts of the body (Barrett 2002; Armbruster *et al.* 2006), an argument first proposed by Darwin (1877). Thus, pollinator behaviour becomes prominent in the selection and maintenance of stylar polymorphisms, although unequivocal evidence for this is scarce, being experimental (Stone & Thomson 1994; Lau & Bosque 2003; Pérez-Barrales & Arroyo 2010; Simón-Porcar *et al.* 2014) and comparative in populations and species with different pollinator arrays (Arroyo & Dafni 1995; Pérez-Barrales *et al.* 2006; Santos-Gally *et al.* 2013b). The abundance and distribution of floral morphs (Thompson *et al.* 2003; Cesaro & Thompson 2004; Brys *et al.* 2007) and the close fit between flowers and pollinators (Massinga *et al.* 2005; Armbruster *et al.* 2006) are critical for the maintenance of the polymorphism. In contrast, less is known about the influence of floral display, in terms of morphological and colour differences, on the reproductive success of the morphs (Brys *et al.* 2004; García-Robledo & Mora 2007; Brys & Jacquemyn 2010), or the influence of floral fragrances for the pollination of the floral morphs (Ashman 2009).

Narcissus papyraceus is a style dimorphic bulb geophyte from the SW Mediterranean, where it blooms in mid-winter. It is a self-incompatible species, although intra- and inter-morph crosses are fully compatible (Arroyo *et al.* 2002; Simón-Porcar *et al.* 2015a). Flowers are white and sweet-scented, with a wide corona and a long and narrow floral tube, at the bottom of which nectar accumulates. This combination of traits fits well with an apparent nocturnal moth pollination syndrome (Faegri & van der Pijl 1979), and it has been suggested that the floral corona functions as a scent-producing organ (Vogel & Müller-Doblies 1975). Flowers are either long- (L) or short-styled (S; Figure S1), the stigma of the latter being placed deep inside the floral tube below the anthers, in a way that only long-tongued pollinators are able to deliver pollen to the stigmas, whereas L-flowers can be reached by long- and short-tongued pollinators (Simón-Porcar *et al.* 2014). Within its geographic range in the southern Iberian Peninsula and northwest Africa, *N. papyraceus* populations are either dimorphic (i.e. both L- and S-morphs are present in the populations in areas with mild winter climate close to the Strait of Gibraltar), or L-monomorphic, a variation that seems to be associated with the activity of the pollinator fauna in the northern range limit of the species. Specifically, the visitation rate of nectar-seeking long-tongued insects in dimorphic populations (mostly diurnal and nocturnal Lepidoptera, including *Macroglossum stellatarum* and *Autographa gamma* as common visitors) approximately doubles that of short-tongued pollen-collecting insects. In contrast, visitation rate of short-tongued insects (with the syrphid *Eristalis texax* and species of *Amegilla* bees as main visitors) is five times greater than long-tongued insects in L-monomorphic populations (see Pérez-Barrales *et al.* 2007; Santos-Gally *et al.* 2013b for detailed pollinator censuses and species list). Arroyo *et al.* (2002) described a gradual loss of S-plants across populations and proposed that such pattern was driven by the increasing presence of short-tongued insects, which cannot deliver pollen

to short stigmas (Simón-Porcar *et al.* 2014). Pollinator variation across temporal and spatial scales is common in the Mediterranean (Herrera 1988; Petanidou *et al.* 2008). In this respect, *Narcissus* has been shown to be an ideal Mediterranean group to assess associations between varying pollinator fauna and floral traits across lineages and populations (Pérez-Barrales *et al.* 2006; Santos-Gally *et al.* 2013a; Pérez-Barrales *et al.* 2014), although it remains unknown the functional significance of these associations.

The present study aimed at identifying the importance of the floral corona of *N. papyraceus* for pollinator attraction in order to evaluate its influence on seed production. To this end, the colour (i.e. reflectance spectra) of the corona and tepals was described, and volatile organic compounds (VOCs) were measured in intact flowers and flowers with trimmed coronas from both morphs, to understand the potential role of the corona to attract pollinators visually, or by means of scent production. The effect of the corona on the pollination of L- and S-flowers was further studied under the natural pollination environment of the dimorphic and L-monomorphic region in a factorial design, where the floral corona was removed from plants distributed individually or in groups. Specifically, we wished to answer the following questions: (i) how does corona removal affect seed production of L- and S-flowers, and (ii) what is the importance of distance among groups of plants and group size for seed production under two different pollinator environments with different flight and visitation patterns, be these represented by mostly long-tongued nectar-seeking insects and short-tongued pollen collecting insects? As small variations in range and flowering period can influence the pollinator community in the Mediterranean, our general purpose was to understand whether different pollinator arrays affect plant fitness differently depending on flower architecture in a winter-blooming Mediterranean species.

MATERIAL AND METHODS

Study species and populations

Most of populations of *N. papyraceus* occur in the region of the Strait of Gibraltar (SW Iberian Peninsula and NW Morocco). Populations are large and dimorphic in the core region of the Strait of Gibraltar, while smaller and L-monomorphic in the northern range margin (Arroyo *et al.* 2002). This geographic pattern of variation in the polymorphism follows a clinal gradient, from isoplethy (equal morph ratio) to anisoplethy (L>S) and to L-monomorphism (Figure S2). Flowering season usually starts in mid-November in isoplethic populations from coastal areas near the Strait of Gibraltar, and ends in late March in L-monomorphic populations from inland and highland areas. An individual flower blooms for 1 or 2 weeks, depending on pollination success, and an entire population blooms for approximately 6–8 weeks (Arroyo *et al.* 2002). Flowers are displayed in umbels of five to 15 flowers, but only the top seven to eight flowers, at most, produce fruits. Thus, only these were used in the experiments described below. Of the 26 compounds identified in the floral scent (Dobson *et al.* 1997), the most abundant is trans- β -ocimene (70%), followed by benzyl acetate and indole. Short-tongued diurnal pollinators occur in L-monomorphic and dimorphic populations, but visitation rate is higher in the former, with 0.8 and 0.1 visits·min⁻¹

respectively. Long-tongued diurnal and nocturnal insects are relatively more frequent in dimorphic populations (Pérez-Barrales *et al.* 2007; Santos-Gally *et al.* 2013b), the latter being particularly important for seed production of S-flowers (Pérez-Barrales & Arroyo 2010).

Description of floral colour and VOCs

The colour of *N. papyraceus* flowers was characterised using an Ocean Optics USB-2000 spectrometer and a Top Sensor System Deuterium-Halogen DH-2000 lamp as a standardised light source (DT-MINI-GS-2). Reflectance was measured as the proportion of a standard white reference tile (WS-1 SS; Ocean Optics, Duiven, The Netherlands). A coaxial fibre cable (QR-400-7-UV-VIS-BX; Ocean Optics) was used for all measurements, and the distance between the sample and the measuring probe was held constant. The angle of illumination and reflection was fixed at 45°. Spectral data were processed with the software Spectrasuite (version 10.4.11; Ocean Optics) and calculated in 5-nm wide spectral intervals over the range 300–700 nm, which is the visual range for most pollinators. Reflectance was measured in the external and internal tepals, and in the outer and inner part of the corona (Figure S1), using the first flower from 24 different individual plants. As control measure, reflectance was measured in five anthers and in the stem of 10 plants.

To quantify floral VOCs, 10 plants (five of each morph) were collected from a dimorphic population and transported to the University of Seville greenhouse facilities. The top two flowers were used to extract floral VOCs, with one flower as control (corona left intact) and the other with manipulation (corona trimmed). This was replicated across the 10 individual plants, with treatment randomised in relation to flower position as well as flower anthesis (VOC collection was done in 1 to 3-day-old flowers). Fragrances were collected between 19:00 and 20:00 h using dynamic headspace sorption and enclosing a single flower within a microwave-safe oven bag (ITS[®]; Nagelpoelweg, The Netherlands). Volatile compounds were adsorbed for 15 min on a trap of 5 mg Porapak Q (Mesh size 80/100; Alltech, Deerfield, IL, USA) connected to a battery-operated vacuum pump (SKC, Eighty Four, PA, USA), which drew the air through the bag and trapped VOCs at a constant rate of ca. 150 ml·min⁻¹. Filters were sealed and stored at -20 °C until elution with 50 µl hexane:acetone mixture (10:1) in the laboratory. All floral odour samples were analysed with a gas chromatograph (GC; Agilent 6890N, Agilent Technologies, Palo Alto, CA, USA) and a mass selective detector (MSD). The separation of VOCs was conducted with an HP5 column (5% phenyl-methylpolysiloxane, 30 m × 0.32 mmØ × 0.25 mm film thickness; Agilent), with helium as carrier gas. An aliquot of 1 µl of the odour sample was injected splitless at a temperature of 50 °C (3 min) followed by opening of the split valve and programming to 250 °C at a rate of 5 °C·min⁻¹. Chromatogram outputs were recorded with the Chemstation program (Agilent). VOCs were identified by comparing their mass spectra and retention times with those in the NIST library and those of authentic standards. To calculate absolute amounts from peak areas, a response factor was used, which was calculated as the mean response of 100 ng of three reference compounds (cresol, limonene, 3-hexen-1-ol) in the GC-MS (but this approach did not always allow identifying the type of

isomer present). Because we only compared the amount of VOCs between morphs with and without corona, we did not calculate the absolute amounts of compounds. Instead, peak areas of total ion chromatograms were used and ln(1 + x) transformed for comparative analyses.

Corona removal, pollen limitation and seed production of L- and S-plants in experimental populations

The experiment was conducted in the dimorphic region (Sieras de Algeciras cork oak open woodlands, Cádiz province, 36°15'15" N, 5°21'31" W, #2 in Figure S2) and the L-monomorphic region (Aznaalcázar pine woodlands, Seville province, 37°15'47" N, 6°13'12" W, #1 in Figure S2). Between December 2004 and January 2005, L- and S-plants with similar floral bud development were collected from a large dimorphic population (#3 in Figure S2). Plants were transferred to pots with a mix of peat and perlite (3:1) and watered daily in the greenhouse of the University of Seville (Spain). Plants were randomly assigned to all combinations of treatments in a factorial design to assess the effects of floral morph (L and S), corona manipulation (intact or removed with fine scissors immediately after flower opening) and limited compatible pollen, with plants in solitary stands or in groups. Groups included three different individual plants of the same morph to avoid a complex experimental design (the experiment was not designed to discriminate assortative and disassortative mating on seed production; see Pérez-Barrales & Arroyo 2010; Simón-Porcar *et al.* 2014 for specific tests). The experiment in the dimorphic and monomorphic region started in December 2004 and January 2005, respectively (sites #2 and #1 in Figure S2). Each treatment combination included three replicates, which were placed randomly in the field at least 150 m apart from one another to reduce pollen flow among plots. Presumably, this is a conservative distance, as shown for *Silene* species pollinated by similar nocturnal and diurnal pollinator arrays, where pollen flow is negligible at distances >80 m (Barthelmess *et al.* 2006; Barluenga *et al.* 2011). In total, 96 plants were collected to conduct the experiment, using 48 plants per region, equally distributed per morph. Prior to the start, the areas selected to set the experiment were inspected (ca. within 2–3 km range) to ensure that there were not naturally co-occurring populations to avoid uncontrolled pollen flow. Experimental plots were left in the field until all flowers withered, and plants were taken back to the greenhouse for fruit maturation to avoid fruit loss. Previous experiments have shown that *N. papyraceus* plants resist well manipulations during their development (Arroyo *et al.* 2002; Pérez-Barrales & Arroyo 2010). Mature fruits were collected, and seed number per flower counted under a stereomicroscope.

Experimental control of the effect of corona manipulation on seed production

Between December 2005 and January 2006, the potential confounding effects of corona manipulation on seed production (e.g. whether damaged flowers produce less seeds) were tested in a population from the dimorphic area (#3 in Figure S2). Thirty patches with at least four plants (*i.e.* two from each morph) were randomly selected and manipulated in such a way that the corona was removed in the top one to three

flowers in one plant per morph, and left intact in the other two plants. To control for the possible pollinator attraction role of the corona, all flowers were supplemented with pollen from other plants. In total, 60 plants per morph were used in this experiment. After 6 weeks, fruits were harvested and seeds counted as described above.

Statistical analyses

To identify differences in reflectance in the corona and tepals, ANOVA was used to compare the parameters hue (largest reflectance value obtained per trait) and brightness (sum of the reflectance values retrieved within the 300–700 nm range; Montgomerie 2006). MANOVA was used to compare VOC emission between treatments (corona present and removed) nested within plants, where morph was included as a fixed factor (due to sample size limitations, plants were not nested within morph). To evaluate the effect of corona removal and plant group size on seed production, generalised linear models (GLM) were performed on the data collected from the experimental settings in the dimorphic and monomorphic regions. These analyses assessed the significance of main effects (morph, corona manipulation and group size) and the interaction effects morph \times corona manipulation, morph \times group size and corona manipulation \times group size. To evaluate the possible effect of corona manipulation on seed production, a two-way full factorial GLM was used, with corona manipulation and morph as fixed factors and seed production as response variable. In this case, a generalised estimating equation (GEE) was applied, setting the correlation structure AR(1), and including patch as repeated measured to account for lack of independence in seed production within patch. Because overdispersion was detected in the response variable seed production, all GLM analyses were conducted with negative binomial error distribution, and the predicted means and SE were retrieved from the models. In all cases, *post-hoc* comparisons (least significance difference) were used when statistically significant effects were detected. The analyses on VOCs were conducted in R version 3.2.1 (R Core Team 2016), whereas the GLMs were run in SPSS version 23 for Mac (IBM, Amok, NY, USA).

RESULTS

Description of floral colour and VOCs

Reflectance was measured every 5 nm over the range of 300–700 nm. On average, all floral parts measured peaked at 450 nm and displayed a similar profile (Fig. 1). Reflectance was higher in the inner and outer tepals, with values around 65% and 61% (maximum reflectance), respectively, across the wavelength range. The measures taken in the external and internal parts of the corona presented a maximum reflectance of 62% and 46%, respectively, after which reflectance declined (Fig. 1). The results of ANOVA showed that traits differed in hue and brightness (ANOVA: $F_3 = 3.094$, $P = 0.031$; $F_3 = 2.903$, $P = 0.039$, respectively). The *post-hoc* analyses revealed that hue values for the internal parts of the corona were significantly lower than those obtained in the external parts, as well as in the inner and outer tepals (all comparisons $P < 0.05$). Hue values were also lower in the external parts of the corona compared to the inner

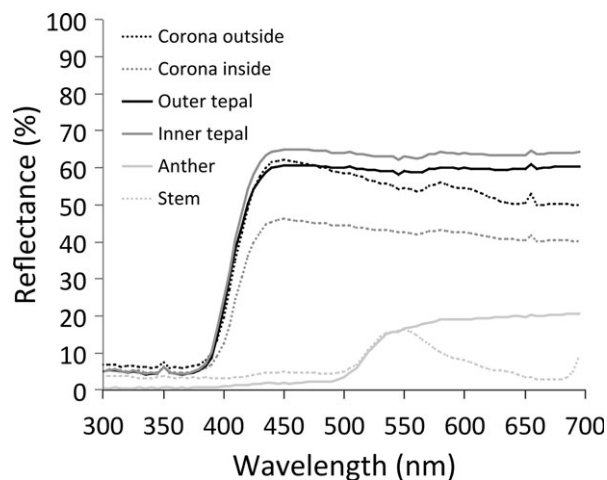


Fig. 1. Spectral reflectance of *Narcissus papyraceus* flowers through UV and human vision wavelengths. The inner (solid grey line) and external (solid black line) tepals, and the external part of the corona (dotted black line) all peaked at ca. 450 nm with a reflectance around 60%, while the internal part of the corona (dotted grey line) peaked at the same wavelength but at ca. 45% reflectance.

and outer tepals (all comparisons $P < 0.05$); in contrast, the inner and outer tepals did not differ in hue (Table 1). For brightness, the *post-hoc* analyses revealed that only the inner corona displayed significantly lower values than the other traits measured (all comparisons $P < 0.05$; Table 1).

A total of 22 VOCs were detected, of which 10 VOCs were identified (Fig. 2). The two dominant compounds were the monoterpene cis- β -ocimene and the aromatic benzyl acetate (components 5 and 8 in Fig. 2). Overall, there were not statistically significant differences in the production of the VOC components between morphs and in relation to the removal of the corona ($F_1 = 0.64$, $P = 0.750$; $F_1 = 2.72$, $P = 0.295$, respectively).

Corona removal, availability of compatible pollen and seed production of L- and S-plants in experimental populations

The analyses to evaluate the effect of corona manipulation and group size on seed production revealed different patterns. In both regions, L-flowers produced more seeds than S-flowers (Table 2, Fig. 3a), and plants in groups produced on average more seeds per flower than solitary stands (Fig. 3c). In contrast, the effect of corona manipulation was only statistically significant in the L-monomorphic region (Table 2), although

Table 1. Mean and SD of reflectance parameters measured in the corona and the tepals of *Narcissus papyraceus* flowers.

trait	hue (%)		brightness	
	mean	SD	mean	SD
corona - external part	62.5	25	3355	1560.7
corona - internal part	46.4	27.4	2583	1717.6
outer tepal	61.3	24.2	3581	1588.2
inner tepal	65.5	24.6	3822	1268.6

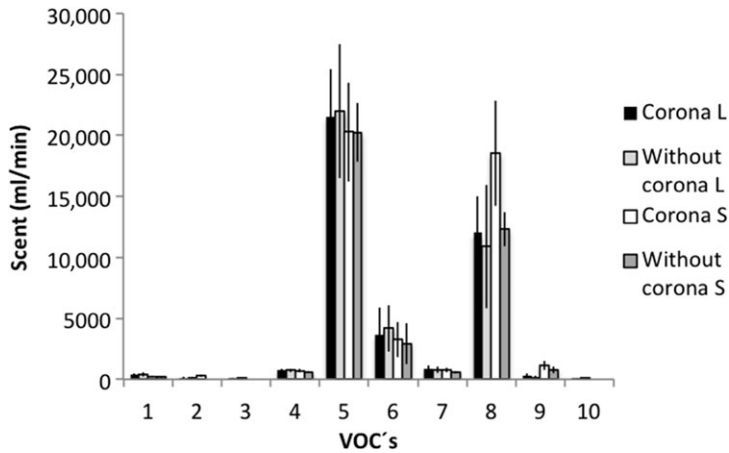


Fig. 2. Mean \pm SE of VOCs emitted by long-styled (L) and short-styled (S) morphs of *Narcissus papyraceus*. VOCs numbers from 1 to 10 represent the components identified as follows: 1, Pentamethyl heptene (3.33), 2, RT3.47, 3, RT3.81, 4, trans-b-Ocimene (3.9), 5, cis-b-Ocimene (4.16), 6, p-Cresol (4.74), 7, Terpinolen (5.15), 8, Benzyl acetate (5.67), 9, Methoxy cresol (6.1) and 10, Tetradeceane. Experimental treatment refers to flowers where the corona was removed (treatment) while in the other remained intact (control).

Table 2. Results of the GLM to estimate the effect of morph (L- and S-flowers), corona manipulation (removed and intact) and group size (one plant and three plants) on seed production of *Narcissus papyraceus* flowers in a natural region with mainly long-tongued pollinators (style dimorphic region) and short-tongued pollinators (style monomorphic region, see Material and Methods for details).

fixed factors	dimorphic region			monomorphic region		
	Wald chi-square	df	P	Wald chi-square	df	P
morph	9.874	1	0.002	3.687	1	0.055
corona	2.507	1	0.113	3.862	1	0.049
group	30.289	1	<0.001	81.329	1	<0.001
morph \times corona	3.967	1	0.046	5.199	1	0.023
morph \times group	13.480	1	<0.001	0.598	1	0.439
corona \times group	1.341	1	0.247	0.025	1	0.875

both regions displayed similar patterns (Fig. 3b), where plants bearing flowers with trimmed coronas had lower seed production than those with the corona intact.

The interaction term morph \times corona was statistically significant in the dimorphic and L-monomorphic region

(Table 2). The *post-hoc* comparison revealed that only S-flowers with the corona trimmed experienced a reduction in seed production ($P = 0.04$ and $P = 0.05$ in the dimorphic and monomorphic region, respectively; Fig. 4a and b), whereas seed production remained similar in manipulated and unmanipulated L-flowers ($P > 0.5$ in both regions). The comparisons between morphs revealed that, in the dimorphic region, unmanipulated S-flowers produced less seeds than unmanipulated L-flowers, with differences only marginally significant (Fig. 4a; $P = 0.08$), while seed production remained similar in unmanipulated L- and S-flowers in the L-monomorphic region ($P = 0.89$; Fig. 4b). In contrast, trimmed S-flowers consistently produced less seeds than trimmed L-flowers ($P = 0.001$ in the dimorphic region, $P = 0.002$ in the monomorphic region; Fig. 4a and b).

The interaction term morph \times group was statistically significant only in the dimorphic region (Table 2, Fig. 4c and d). Both L- and S-plants in groups produced more seeds than in solitary stands ($P = 0.021$ for comparison between L-flowers; $P < 0.001$ for comparisons between S-flowers; Fig. 4c). Interestingly, the reduction in seeds per flower was stronger in solitary stands of S-plants than L-plants ($P < 0.0001$). In the

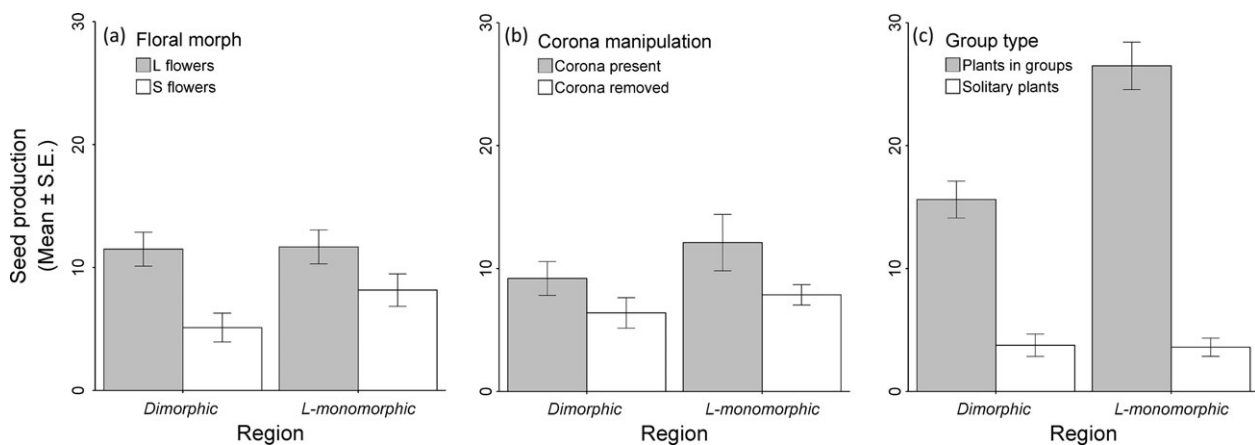


Fig. 3. Predicted mean seed production \pm SE for the main effects floral morph (a), corona manipulation (b) and group size (c) retrieved from the generalized linear model to evaluate the effect of corona removal and group size on the production of seeds in long-styled (L) and short-styled (S) flowers of *Narcissus papyraceus* in a natural dimorphic and monomorphic region.

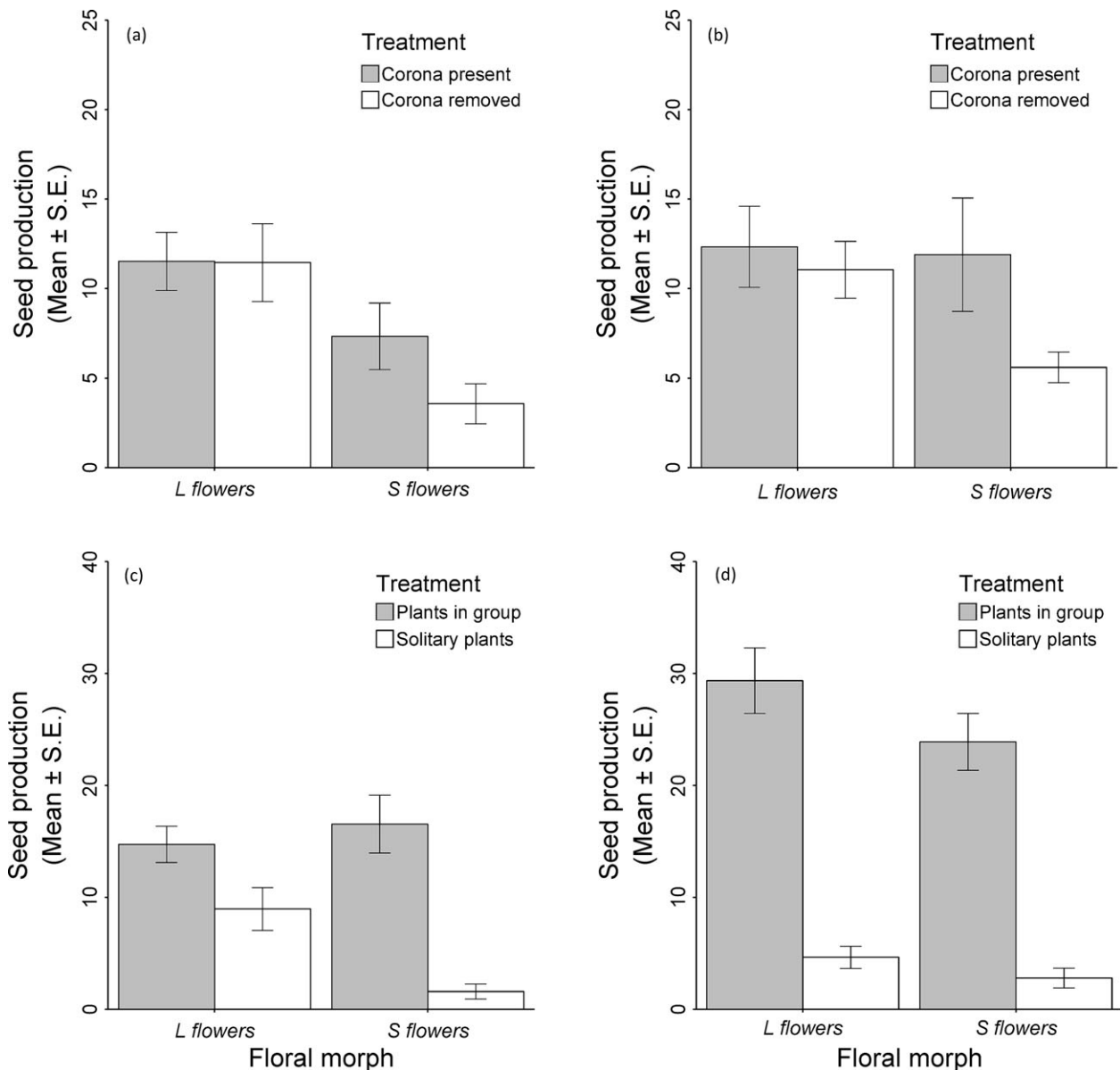


Fig. 4. Predicted mean seed production \pm SE for the interaction effects morph \times corona manipulation (a and b for the dimorphic and L-monomorphic region respectively), and morph \times group size (c and d for the dimorphic and L-monomorphic region respectively) retrieved from the generalized linear model to estimate the effect of corona removal and group size on the production of seeds in long-styled (L) and short-styled (S) flowers of *Narcissus papyraceus* in a natural dimorphic and monomorphic region.

monomorphic region, the patterns between L- and S-plants were consistent, and seed production per flower was lower in solitary stands than in groups (Fig. 4d).

Experimental control of the effect of corona manipulation on seed production

The effect of corona removal on seed production was not statistically significant (Wald $\chi^2 = 0.545$, $df = 1$, $P = 0.46$), but the term 'morph' was (Wald $\chi^2 = 11.60$, $df = 1$, $P = 0.001$). On average, L-plants produced more seeds per flower (11.28 ± 1.03) than S-plants (6.31 ± 0.87). The interaction term morph \times corona was also statistically significant (Wald $\chi^2 = 10$, $df = 1$, $P = 0.002$). Specifically, L-flowers with the

corona removed produced less seeds than those where the corona was left intact (L-flowers with corona removed: 9.55 ± 1.09 ; L-flowers with corona: 13.11 ± 1.71), whereas the opposite trend was detected for S-flowers (S-flowers with corona removed: 8.09 ± 1.35 ; S-flowers with corona: 4.93 ± 0.80).

DISCUSSION

Floral corona as visual and fragrance attractive trait

The reflectance profile of *N. papyraceus* flowers is similar to other species with nocturnal pollination (Raguso *et al.* 2003). Both corona and tepals peaked at ca. 450 nm with similar percentage of reflectance (except for the internal part of the

corona), although reflectance parameters (hue and brightness) were generally larger in the tepals. Emission of VOCs in intact L- and S-flowers was similar, and corona removal did not seem to decrease it. These results should be interpreted cautiously for the following reasons. The extraction methods did not allow us to quantify variation in the rate of production among different floral organs (Burdon *et al.* 2015; Prieto-Benitez *et al.* 2016), or whether compound emission followed circadian patterns (extractions were done between 19:00–20:00 h), which usually affects attraction of nocturnal and diurnal visitors (Balao *et al.* 2011; Burdon *et al.* 2015; Prieto-Benitez *et al.* 2016). In cultivated daffodils and in *N. serotinus*, the compounds released during the day and at night are similar, with slightly higher percentage in the diurnal emission (Dobson *et al.* 1997; Ruíz-Ramón *et al.* 2014). It could be argued that *N. papyraceus* populations with different contribution of long-tongued (diurnal and nocturnal insects) and short-tongued pollinators could be associated with regional ecotypes with regard to compounds emitted, as Dobson *et al.* (1997) detected in populations of *N. assoanus* from the SW and NE of Spain. In a monomorphic population of *N. papyraceus*, those authors identified cis- β -ocimene, trans- β -ocimene, p-cresol and benzyl acetate as the main volatiles, similar to the main components detected in the dimorphic population that we studied. Our data do not allow us to establish direct causal relationships between variation in reflectance and fragrance production with the attraction of specific pollinators, but the variation observed can be interpreted in that context. For example, from a visual perspective, the reflectance profile observed is similar to other species with nocturnal pollinators (Raguso *et al.* 2003), with a close match between the reflectance peak and the range of the receptor peak spectral sensitivity of many insects (reviewed in Briscoe & Chittka 2001), including nocturnal and diurnal Lepidoptera (Raguso & Willis 2002; Kelber *et al.* 2003) and the hawkmoth *Macroglossum stellatarum* (Kelber 1997). The spectral sensitivity of syrphids is also within the range of the peak identified in *N. papyraceus* flowers (Lunau 2014). These flies usually respond to floral patterns of white background with a central yellow spot (Dinkel & Lunau 2001; Lunau 2014), which is the pattern of *N. papyraceus* flowers (Figure S1), as well as fragrance stimuli (Primante & Dotterl 2010). In addition, the main VOCs identified usually elicit a response in the nocturnal *N. papyraceus* visitor *Autographa gamma* (Plepys *et al.* 2002), and in other nocturnal and diurnal visitors (Jürgens *et al.* 2014). To better understand whether variation in reflectance and fragrance production in *N. papyraceus* affects pollinators, future research should incorporate behavioural and physiological responses of different pollinator groups. However, the small variation in fragrance after corona removal and in reflectance, seems to suggest that this organ is probably less important for pollinator attraction than tepals. Further work in other *Narcissus* species with a high contrast between corona and tepal colour could produce different results.

Effect of corona manipulation and patch size on seed production

The results of the manipulation of the corona revealed similar patterns of variation in the dimorphic and monomorphic regions. Corona removal had a detrimental effect on the pollination of S-flowers, with ca. 50% reduction in seed production

in both regions (Fig. 3), while production of seeds was similar in L-flowers with intact and trimmed coronas. In our experiment, it is difficult to interpret whether the removal of the corona decreased attractiveness and therefore pollinator visitation (see Discussion above). Instead, the corona might be important for pollinator positioning to facilitate a close mechanical fit with flowers (Armbruster *et al.* 2006; Muchhala & Potts 2007), which becomes more important for long-tongued insect-pollinated species (Ellis & Johnson 2010; Newman *et al.* 2015). In *N. papyraceus*, while both long-tongued and short-tongued pollinators deliver pollen to L-stigmas, S-flowers require the visit of long-tongued insects to successfully deposit pollen on the stigmas (Simón-Porcar *et al.* 2014). Furthermore, in dimorphic populations, the relative contribution of nocturnal moths to seed production is more important than that of diurnal pollinators, particularly for S-flowers (Pérez-Barrales & Arroyo 2010). Whether it affects attraction or mechanical fit, the fact that only trimmed S-flowers experienced a reduction in female fitness suggests that the corona is important for long-tongued nectar-seeking insects.

We conducted an experiment to assess the possible confounding effects of corona removal on seed production (*e.g.* reduction caused by damage or resource re-allocation), but the control experiment showed that this was not the case. Hence, the results obtained in the experimental plots are not an artefact of the manipulation. Although there were differences between morphs in the control experiment, these did not contradict our findings. Instead, the results might be related to our unequal ability to conduct hand-pollinations in L- and S-flowers (*e.g.* stigmas in L-flowers are more exposed than those in S-flowers).

The purpose of grouping plants in experimental plots allowed us to estimate the importance of the incompatibility system in *N. papyraceus* and distance between compatible plants. Consistently, solitary plants produced fewer seeds than grouped plants. Grouped plants probably increased floral display and pollinator attraction (Harder & Barrett 1995; Harder & Johnson 2005). Presumably, visitation rate and out-crossed pollen arrival was lower in solitary stands (Jennersten & Nilsson 1993; Engel & Irwin 2003). It was remarkable to find that the interaction effect group \times morph was statistically significant only in the dimorphic populations. Specifically, differences in seed production between S-plants in solitary stands and in groups were larger than those observed in the same comparison for L-plants. Disassortative pollen transfer mediated by long-tongued pollinators is relatively more important for S-plants (Thompson *et al.* 2003; Cesaro & Thompson 2004; Simón-Porcar *et al.* 2014; but see Pérez-Barrales & Arroyo 2010), whereas female fitness of L-plants can be achieved regardless of the morph of the pollen donor or pollinator type (Pérez-Barrales & Arroyo 2010; Simón-Porcar *et al.* 2014). In general, while pollination events become negligible with distance, nocturnal insects are more efficient at pollen dispersal than diurnal insects in these pollinator arrays (Schulke & Waser 2001; Barthelmess *et al.* 2006; Barluenga *et al.* 2011). In the dimorphic region, the relatively higher contribution of nocturnal insects to female fitness (Pérez-Barrales & Arroyo 2010) suggests that these might have facilitated pollen dispersal to solitary plots from nearby plots (ca. 150 m apart).

It was unexpected to find that experimental plots of S-flowers produced seeds in the monomorphic region. The main

visitors here are short-tongued insects, which are inefficient in the pollination of S-flowers; instead, they promote self-pollination, which reduces seed production (Simón-Porcar *et al.* 2014). Discordance from our expectations refers only to long-tongued pollinators. Temporal variation in the pollinator community can affect female fitness (Herrera 1987, 1988). This might be the case in *N. papyraceus* populations, although the variation refers to insect visitation rate and not the relative abundance of long-tongued *versus* short-tongued insects in dimorphic and L-monomorphic populations (Pérez-Barrales 2005). In the present study, it was not feasible to incorporate observations of pollinators in our experiments because *N. papyraceus* is a winter-blooming species, with very low visitation rates (Pérez-Barrales *et al.* 2007; Santos-Gally *et al.* 2013b), particularly in isolated or small groups of plants, as those used in our experiments.

Role of the corona in the function of style polymorphism in *Narcissus*

The form and size of the corona, together with the floral tube, have been proposed as important traits for the evolution and maintenance of style polymorphism in *Narcissus* (Graham & Barrett 2004; Pérez *et al.* 2004; Barrett & Harder 2005; Pérez-Barrales *et al.* 2006; Santos-Gally *et al.* 2013a). Species pollinated by large bees usually present medium size cup-shaped coronas, which probably allows a close fit between bees and flowers. Other species with larger and wider coronas and floral tubes provide basking sites with high temperatures inside the flower, where insects move freely (although these species are style monomorphic *Narcissus* sect. *Pseudonacissi* and *Bulbocodium*; Herrera 1995). Experimental manipulations have helped quantifying the adaptive value of flower traits in the attraction of pollinators (Borges *et al.* 2003; Armbruster *et al.* 2005). Our study provides experimental evidence for the importance of the floral corona in a style dimorphic species by measuring its effects on seed production, although it remains to be tested whether the corona (and probably the tepals) influence pollinator behaviour visually and by means of fragrance emission. Despite this limitation, one of the key results, *i.e.* the substantial reduction in seed production in S-flowers with trimmed coronas, can only be explained in terms of pollination by long-tongued pollinators (mostly moths and hawkmoths). Corona was less important for female fitness in L-flowers. Probably, short-tongued syrphid flies use different combinations of floral cues and equally pollinate L-flowers with or without coronas (see Discussion above). Here we provided a mechanistic explanation to former results on the contribution of contrasting pollinator groups to female fitness (Pérez-Barrales & Arroyo 2010; Simón-Porcar *et al.* 2015b).

Similar to *N. papyraceus*, other *Narcissus* species present pollinator shifts (Arroyo & Dafni 1995; Pérez-Barrales *et al.* 2006;

Santos-Gally *et al.* 2013a), which are usually associated with changes in the perianth (Pérez-Barrales *et al.* 2006, 2007, 2009, 2014; Santos-Gally *et al.* 2013a). Moreover, variation in corona morphology within and between species has been used as a taxonomic trait (Aedo 2013). Given its importance for the pollination of S-flowers, it would be interesting to compare the direction and strength of selection on the floral corona between style polymorphic species (*i.e.* species with L- and S-flower) and species with only one floral morph (usually the L-morph in *Narcissus* species). Together, the variation in morphology of the corona and chemical composition of fragrances offer a promising avenue for further investigating how these traits attract different functional pollinators and affect plant fitness. This will add more insights to the ecological and evolutionary mechanisms that maintain the stylar polymorphism in this Mediterranean winter geophyte with such a large variation in pollinators.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Style dimorphism in *Narcissus papyraceus*, with stigmas of long-styled flowers (left image) and short-styled flowers (right image) above and below the anther level respectively.

Figure S2. Geographic range of populations of *Narcissus papyraceus* with areas of long-styled monomorphism (L), anisoplethic dimorphism (L>S) and isoplethic dimorphism (L=S) depicted by dashed lines. Experimental populations: 1, Aznalcazar (Sevilla prov.); 2, La Alcaidesa (Cádiz prov.); 3, Bolonia (Cádiz prov).

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