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NATURAL SELECTION ON FLORAL TRAITS THROUGH MALE AND FEMALE FUNCTION IN WILD POPULATIONS OF THE HETEROSTYLOUS DAFFODIL *NARCISSUS TRIANDRUS*

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Measurements of natural selection in hermaphrodite populations require the analysis of performance through both female and male sex functions. Here, we investigate selection on three floral traits: flower number, flower length, and corona width through both sex functions in natural populations of the tristylous daffodil *Narcissus triandrus*. Selection through female function was examined in six populations, and in two of these we also estimated male selection gradients using multilocus microsatellite genotyping of parents and offspring. We detected significant directional selection for flower number through female function, and significant stabilizing selection for corona width and flower length through male function. Variation in male reproductive success was strongly influenced by the distance between mates and was significantly higher than variation in female reproductive success in one population, a result consistent with Bateman's principle. However, variation through both sex functions was similar in the other population and there was a significant negative correlation between female and male fitness indicating sex-specific trade-offs in reproductive success. Selection on floral design in *N. triandrus* was stronger through male than female function probably because floral morphology plays an important role in promoting effective cross-pollen transfer in populations of this heterostylous species.

KEY WORDS: **Female fitness, floral evolution, male fitness, microsatellite markers, selection gradients.**

Measuring natural selection on floral traits is central to understanding floral diversification and the maintenance of sexual polymorphisms in animal-pollinated plants. A growing number of studies have detected selection on floral traits including those involved in pollinator attraction, such as flower color (Stanton et al. 1986; Gigord et al. 2001; Jones and Reithel 2001), flower number (Conner and Rush 1997; Vaughton and Ramsey 1998; Benitez-Vieyra et al. 2006), and flower size (Conner et al. 1996; Vaughton and Ramsey 1998; Morgan and Conner 2001; van Kleunen and Ritland 2004; Fishman and Willis 2008), as well as traits that function to improve the mechanical fit between flowers and pollinators (Nilsson 1988; Campbell 1989, 1991; Johnson and Steiner 1997; Alexandersson and Johnson 2002). Studies of outcrossing species generally focus on variation in female fitness, because of the difficulty of measuring male fitness in plant populations (reviewed in Herrera et al. 2006). However, most flowering plants are hermaphroditic and, on average, obtain half of their fitness through male function. Consequently, measuring variation in fitness through male function should be a critical element of any study of selection on floral traits and inferences on floral evolution that ignore this component of fitness should be cautiously evaluated.

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Indirect measures of male fitness, such as pollen removal (Nilsson 1988; O'Connell and Johnston 1998; Maad and Alexandersson 2004) or insect visitation rate (Inoue et al. 1995; Conner and Rush 1996), are often used in lieu of direct measures of male fertility, but in many cases provide biased estimates (Snow and Lewis 1993; Klinkhamer et al. 1994). The recent use of hypervariable molecular markers and development of statistical techniques to assess paternity have enabled direct estimates of variation in male fitness in plant populations (e.g., Conner et al. 1996; Kobayashi et al. 1999; Smouse et al. 1999; Morgan and Conner 2001; van Kleunen and Ritland 2004; Wright and Meagher 2004). However, despite the utility of these methods for assessing paternity and outcrossed siring success, there is a general paucity of studies that have measured natural selection through male function, particularly in wild plant populations in their native ranges (reviewed in Conner 2006; Morgan 2006). Investigations that have directly measured selection through both male and female function have often found contrasting patterns of selection (Devlin and Ellstrand 1990; Conner et al. 1996; Kobayashi et al. 1999; Elle and Meagher 2000; Morgan and Conner 2001; Ashman and Morgan 2004; van Kleunen and Ritland 2004; Wright and Meagher 2004) raising the question as to why patterns of selection through male and female function may differ.

The application of sexual selection theory (Bateman 1948) to hermaphroditic plants (Janzen 1977; Willson 1979) has led many researchers to conclude that attractive floral displays evolve mainly in response to selection through male function (Bell 1985; reviewed in Burd and Callahan 2000). Specifically, the "male function hypothesis" assumes that resources rather than pollen delivery limit female fitness and that selection on floral characters is stronger through male than female function. However, empirical support for this hypothesis is limited (Wilson et al. 1994; Ashman and Morgan 2004) and there is growing evidence that the ecological and reproductive context of populations (e.g., environmental conditions causing variation in pollen limitation of female fertility) is also important for determining the nature of selection through male and female function (Wilson et al. 1994; Ashman and Diefenderfer 2001; Caruso et al. 2005; Fishman and Willis 2008). Nevertheless, few studies have used direct measurements of male and female selection gradients for attractive traits, such as flower size and number, to critically evaluate the "male function hypothesis" (but see Ashman and Morgan 2004).

Increased flower size and number enhance attractiveness to pollinators and components of floral display and design may therefore be the targets of directional selection. However, floral morphology not only functions in attraction but also influences the physical interaction between pollinators and reproductive organs once animals arrive at flowers. For example, in many species flower size and shape are considered to be functionally optimized for matching flowers to their predominant pollinators to facilitate precise pollen transfer among plants (Darwin 1862; Stebbins 1970; Cresswell 1998). If this is true, components of floral architecture are likely to be commonly under stabilizing selection. In addition, other mechanisms, such as nonpollinator agents of selection (Strauss and Whittall 2006), or trade-offs among reproductive traits can also result in stabilizing selection through either sex function (Morgan and Conner 2001). However, despite some evidence that stabilizing selection on floral traits may be relatively common in plant populations (Cresswell 1998), this form of selection has rarely been measured in natural populations visited by native pollinators.

Narcissus triandrus is a diminutive, outcrossing hermaphroditic daffodil native to the Iberian Peninsula, pollinated by bees (predominantly *Anthophora* and *Bombus*). Geographical variation in flower size and number implicate morphological differentiation among populations in response to local environmental conditions (Barrett et al. 2004; Hodgins and Barrett 2008a). The species is tristylous with populations commonly containing the three style morphs: long-, mid-, and short-styled, (hereafter referred to as L-, M-, and S-morphs) although dimorphic populations missing the M-morph are characteristic of the NW portion of the species range (Barrett et al. 2004). *Narcissus triandru*s possesses a late-acting ovarian self-incompatibility system that permits both intramorph and intermorph mating (Barrett et al. 1997; Sage et al. 1999). Because these two classes of cross-pollination are equally fertile, floral morphology in *N. triandrus* populations plays a more significant role in governing mating patterns and fertility than in most heterostylous species, which typically possess heteromorphic incompatibility. Intramorph compatibility results in asymmetrical mating and biased morph ratios in *N. triandrus* populations (Hodgins and Barrett 2008b).

In common with other animal-pollinated species (Nilsson 1983, 1988; Campbell 1989, 1991; Benitez-Vieyra et al. 2006), variation in flower size and shape in *N. triandrus* is likely to have important fitness consequences through its influence on pollen dispersal. However, little is known about how these floral characters influence cross-pollen transfer, mating, and the maintenance of sexual polymorphism in heterostylous populations. Flower size and shape in *N. triandrus* is strongly influenced by the corona, a tubular structure that functions to position visiting bees during pollination. Only *N. triandrus* and *N. albimarginatus,* the two heterostylous species of *Narcissus,* have both deep coronas and long floral tubes (Graham and Barrett 2004). It has been proposed that this morphological association may have been the structural innovation that resulted in the evolution of heterostyly in *Narcissus* (Barrett and Harder 2005). The corona forces bees to enter the flower in a stereotypical manner and the depth of the corona and floral tube creates vertical space for the spatial separation of sex organs enabling the sex-organ reciprocity that is the defining feature of heterostylous plants. Therefore, this floral design probably facilitates segregation of pollen on the pollinator's body and more precise cross-pollen transfer among the floral morphs. If this functional hypothesis is correct we would predict that extensive variation in flower size and shape should lower pollen-transfer efficiency resulting in pollen wastage.

Here, we measure natural selection on floral traits through male and female function in natural populations of *N. triandrus* to evaluate the function of floral traits in a heterostylous species. Our study addressed four specific questions: (1) Is there evidence for phenotypic selection on floral traits of *N. triandrus*? To address this question, we examined linear and nonlinear forms of selection on three traits: flower length, corona width, and flower number. In our analysis we incorporated interplant distance into statistical models for the estimates of male selection gradients to improve power and to assess the degree of near-neighbor mating. (2) Are there differences in the patterns of selection through male and female function? The "male function" hypothesis predicts stronger selection on flower size and number through male than female function. (3) Is variation in reproductive success through male function greater than through female function, as predicted by Bateman's principle? (4) Is there evidence for a negative correlation between female and male fitness implicating sex-specific trade-offs in reproductive success?

Materials and Methods

STUDY ORGANISM

Narcissus triandrus (Amaryllidaceae) is a spring-flowering geophyte common in the central and northern parts of the Iberian Peninsula. Flowering commences in early March and continues until late April–early May at higher elevations. Flowering plants usually produce a single stem with pale yellow to white flowers, ranging in number from 1 to 9 (mean = 1.6), which last up to 14 days. Flowers are pendulous with reflexed tepals and a narrow floral tube with a prominent corona. Long-tongued bees are the primary pollinators of *N. triandrus*. *Anthophora* spp. are the main visitors in the southern portion of the range but are largely replaced by *Bombus* spp. in the cooler Atlantic zone of northern Spain and Portugal (Hodgins and Barrett 2008a). Pollinator visitation rates are generally low, although, pollen limitation is not common in *N. triandrus* populations (Hodgins and Barrett 2006), probably because of extended floral longevity.

POPULATION SAMPLING

During the springs of 2003 and 2004 we located three dimorphic and three trimorphic populations of *N. triandrus* each containing between 113 and 286 individuals (mean = 160.1). Details of the localities, morph ratios, and population sizes are presented in Table 1. At peak flowering we tagged, mapped, and collected a leaf sample from all flowering individuals in each population. We counted the number of flowers per inflorescence and measured height with a ruler. On the most distal flower (the first to open) on an inflorescence we measured flower length, corona length, width at the mouth of the corona, and upper anther height with digital callipers. The vast majority of individuals were flowering at census and we removed flowering stems from the few individuals that were in bud from the population. For individuals that were past anthesis, we identified their location and collected a leaf sample in case these individuals had already sired seeds. Four to five weeks later, we collected capsules of individuals and counted the number of seeds in each capsule. We also recorded nonfruiting and missing individuals within each population at seed harvest.

DNA EXTRACTION AND GENOTYPING

In the two populations (204, 254) in which we estimated male selection gradients, we extracted total genomic DNA from leaf tissue using the Puregene^{TM} DNA isolation kit (Gentra Systems, Minneapolis, MN). We also extracted total genomic DNA from embryos using the Qiagen DNeasy kit (Qiagen, Mississauga, Ontario, Canada) according to the manufactures instructions for low quantities of DNA (population 204 mean = 5.4 seeds from 41 maternal families; population 254 mean = 6.1 seeds from 50 maternal families). We stored seeds dry in the dark at 4°C. Prior to extraction, we soaked the seeds in distilled water to induce germination and to loosen the seed coat. We then extracted DNA

Table 1. Density, size (*N*), morph frequencies (L-, M-, and S-morph), and location (decimal degrees) for the six Portuguese populations of *Narcissus triandrus* sampled in this study.

Population	Density (N/m^2)	N	L	M	S	Latitude °N	Longitude °W
153 Caria	0.92	124	0.50	0.21	0.29	40.92763	−7.60455
204 Lages Do Outeiro	0.25	138	0.88	0	0.12	40.56791	−7.97905
207 Lamosa	0.48	159	0.48	0.34	0.18	40.87607	−7.61841
208 Ponte Da Barca	0.51	286	0.73	0	0.27	41.80896	−8.37766
245 Mangualde	0.41	141	0.71	0.01	0.28	40.59507	−7.79802
254 Avelal	0.30	113	0.63	0.05	0.32	40.73228	−7.67872

from the germinated cotyledons, or, as germination rates were low, from embryos, which we removed using fine forceps under a dissecting scope. We measured the quality and quantity of all DNA using a mass spectrometer and diluted the DNA to a final concentration of 50 ng/μl for the parental samples and 25 ng/μl for the offspring samples. Samples of low quality were discarded. Consequently, we reextracted parental samples and replaced low-quality offspring samples with other individuals from the same maternal family.

To assess male selection gradients we used five nuclear microsatellite primer pairs NT26, NT63, NT113, NT154, and NT155 to genotype adults and progeny (Hodgins et al. 2007). We performed DNA amplification using the following conditions: 50 ng of genomic DNA in a 25 μl PCR volume, along with 0.1 μM of primer, 1.5mM MgCl$_2$, 0.2 mM of each dNTP, 1.25 U Taq Polymerase (Fermentas), and 1 X PCR buffer with (NH$_4$)$_2$SO$_4$. DMSO was added to NT26 reactions to a final concentration of 5%. The cycling conditions were 4 min initial denaturing followed by 40–50 cycles of: denaturing for 30 sec at 94°C; annealing at 59–63°C (depending on the primer pair) for 30 sec; and extension for 30 sec at 72°C with a 72°C 10 min final extension. We sent the PCR reactions to the Genetic Analysis Facility of The Centre for Applied Genomics (The Hospital for Sick Children, Toronto, ON) for fragment analysis and we conducted size alignments using GeneMapper® ver. 3.5 software (Applied Biosystems, Foster City, CA).

DATA ANALYSIS

Female reproductive success

We estimated selection through female function in all six populations using standardized linear (directional) selection differentials (s), using the GLM procedure in SAS ver. 9.1 (SAS Institute Inc.,). Selection differentials (s) are the univariate regression coefficient between relative fitness (fitness/mean fitness, w) and each of the standardized traits (Lande and Arnold 1983). We conducted all analyses on traits standardized with a mean of 0 and a variance of 1 for each population. This facilitated comparisons of selection acting on traits measured in different units and across different populations. We used the total number of seeds produced per plant, divided by the mean of each population to provide a measure of relative female fitness.

Selection differentials suffer from the fact that selection acting on the trait of interest cannot be disentangled from selection acting indirectly on correlated characters. Therefore, to reduce the confounding effects of indirect selection, we calculated selection gradients using a standard multivariate regression analysis (Lande and Arnold 1983) using the GLM procedure (SAS). Natural selection acting on phenotypic variation is estimated as the regression coefficients of relative fitness (w) on standardized measures of multiple traits thought to affect fitness (z). We report quadratic regression coefficients rather than the adjusted stabilizing/disruptive selection gradients, which are double the quadratic regression coefficients (Lande and Arnold 1983). We removed corona length and upper anther height from the analysis, as flower length was highly correlated with these measures and served as a surrogate for these traits (Table 2). Therefore, we used flower length, corona width, flower number, and plant height for all analyses. Height, flower number, flower length, and corona width, as well as the quadratic components height^2, flower number^2, flower length^2, and corona width^2 were predictor variables in the model. In populations 153 and 245 there was no variation in flower number so for these populations this trait was not included in the model. As seed set was not normally distributed, we assessed significance of the model by log transforming relative seed set and repeating the analysis.

To compare patterns of selection through female function among the six populations we used analysis of covariance (ANCOVA). We analyzed seed set using the MIXED procedure (SAS; Neter et al. 1996) and square root transformed seed set + 1 to improve the normality of the residuals. Height, flower number, flower length, and corona width were included in the model. We incorporated population as a random effect as well as all interactions between population and all other variables in the model. We calculated denominator degrees of freedom for F-tests of fixed effects by Kenward and Roger's approximation (1997). We removed nonsignificant interactions ($\alpha > 0.05$) in a stepwise fashion and

Table 2. Phenotypic correlation matrix of the seven traits measured in population 204 (upper diagonal) and population 254 (lower diagonal) of *Narcissus triandrus*. Significant correlations are in bold.

	Flower number	Height	Flower length	Corona length	Upper anther	Corona width
Flower number	–	**0.63**^{1}	−0.01	−0.03	−0.07	0.10
Height (cm)	**0.22**^{2}	–	0.03	0.10	−0.03	0.10
Flower length (mm)	**0.25**^{3}	**0.29**^{3}	–	**0.48**^{1}	**0.71**^{1}	0.10
Corona length (mm)	0.11	0.11	**0.69**^{1}	–	0.13	0.19^4
Upper anther (mm)	0.12	**0.40**^{1}	**0.71**^{1}	**0.30**^{3}	–	−0.06
Corona width (mm)	**0.27**^{2}	0.09	**0.26**^{3}	**0.33**^{1}	0.09	–

$^1P < 0.001$, $^2P < 0.05$, $^3P < 0.01$, $^4P < 0.1$.

to test the significance of random effects we used likelihood ratio tests. Specifically, for each random effect we ran the MIXED procedure twice, once for the full model including the random effect, and once for the reduced model in which the random effect was not included. Then we subtracted the values of -2 times the log likelihoods for the full and reduced models. Differences in the log likelihoods were tested with a χ^2 test with one degree of freedom. We used a one-tailed test because variances cannot be less than zero (Littell et al. 1996). We repeated ANCOVA with flower number removed from the analysis so all six populations could be incorporated.

Male reproductive success

To estimate male reproductive success we used the ML-based fertility program PatQuest (Meagher 1986; Smouse and Meagher 1994; Smouse et al. 1999). The program uses genotypes of parents and offspring to generate Mendelian segregation probabilities x_{jk} for all potential fathers of each offspring, where x_{jk} is the probability that offspring j is a product of the known maternal genotype and the potential paternal genotype k. The program then calculates the likelihood of the male parentage spectrum (i.e., relative male reproductive success λ_k for each of the k males where $\sum \lambda_k = 1$) as follows:

$$L = \prod_j L_j = \prod_j \Big[\sum_k x_{jk}\lambda_k\Big] \tag{1}$$

and maximizes this likelihood using an iterative algorithm (see Smouse et al. 1999). We performed an analysis to determine if there was significant variation in male reproductive success (λ_k) by testing departure from a uniform distribution using a log-likelihood ratio (LLR), which is asymptotically chi-square distributed with degrees of freedom equal to one less than the number of distinct male genotypes (Smouse and Meagher 1994).

Using PatQuest, we then determined the relation between male reproductive success, floral traits, and distance using a log-linear model (Smouse et al. 1999).

$$\lambda_{jk} = \mathrm{Exp}\Big[\gamma_d d_{jk} + \sum_{\mathbf{i}} \beta_i z_{ik} + \gamma_i z_{ik}^2\Big] \tag{2}$$

Where λ_{jk} represents the fertility of the kth paternal parent on the maternal parent of progeny j and d_{jk} is the log of the distance between each pair of plants in the population (kth paternal parent to the maternal parent of progeny j) and γ_d describes the regression of relative fertility on distance (Adams and Birkes 1991; Smouse et al. 1999). In addition, β_i and γ_i represent the linear and quadratic regression coefficients, respectively for each of i traits (Lande and Arnold 1983) and z_{ik} are the standardized residuals of character measurements, which were obtained by subtracting the population mean of each trait from observed values for each individual and dividing by the standard deviation. This model was used to obtain maximum-likelihood estimates of male selection gradients γ_d, β_i and γ_i from the genetic probabilities x_{jk} and the phenotypic values d_{jk} and z_{ik}. We estimated univariate selection as well as multivariate selection gradients both with distance and without distance in the model. Univariate selection gradients are analogous to Type I ANOVA and often have greater statistical power than multivariate analyses (Elle and Meagher 2000).

We assessed statistical significance of the selection gradients using LLR. Each parameter was assessed one at a time by obtaining the LLR for the model, where the numerator was the estimated likelihood of the reduced model, excluding the parameter, and the denominator was the estimated likelihood of the full model. We then compared the LLR statistic to a chi-square distribution with one degree of freedom. However, significance testing using the parametric method can be problematic (Smouse et al. 1999; Elle and Meagher 2000; Morgan and Conner 2001). Therefore, we tested significance using a pedigree simulation method that determines the fit of the data compared to a null model with constrained parameters (Morgan and Conner 2001). Specifically, for each simulation PatQuest created the same number of progeny for each female as were found in the original dataset, and progeny genotypes were constructed from the maternal and paternal genotypes based on the rules of Mendelian segregation. Males were chosen with a probability in proportion to the male's fertility, using the maximum-likelihood estimations. The program then estimated the parameters for the simulated data in the same manner as above. To determine if a particular parameter was equal to zero, male fertilities and genotypes were generated by replacing the parameter value estimated from the data with zero. The simulated data give an empirical approximation to the distribution of the likelihood-ratio statistic. The proportion of values in the simulated distribution greater than the likelihood ratio of the original data approximates the P-value. To test the significance of variation in male mating success, effects of distance on mating, and univariate selection gradients we performed 1000 simulations, whereas we performed 500 simulations for the multivariate analysis due to the significant computational time required.

Comparison of male and female reproductive success

To compare male and female reproductive success for the two populations in which male fitness estimates were available (populations 204 and 254), we determined the total seed set for each individual and divided by the sum for each population to obtain the female reproductive success of the jth individual, λ_j. This estimate of female fertility is comparable to the one obtained from the paternity analysis (λ_k) (Elle and Meagher 2000). As Bateman's principle predicts greater variance in male than female fitness, we compared the variance in reproductive success between male and female function in both populations using Levene's test of

Table 3. **Standardized selection differentials (*s*) through female function in six populations of *Narcissus triandrus*. We calculated the selection differentials using relative fitness (w), and tested the statistical significance using a log ($w + 1$) transformation to improve normality of the residuals. Analyses were done using standardized trait values. Effects in bold are significant at $P < 0.05$. The standard errors for the selection differentials are shown.**

Trait	Population					
	P153	P204	P207	P208	P245	P254
	s	*s*	*s*	*S*	*s*	*s*
Flower number	—	0.16±0.08	**0.29±0.09**^{1}	**0.32±0.06**^{2}	—	**0.69±0.15**^{2}
Height	0.03±0.19	0.14±0.08	0.03±0.09	**0.29±0.06**^{2}	0.57±0.24	0.32±0.16
Flower length	0.01±0.19	0.07±0.09	0.03±0.09	0.06±0.07	0.14±0.25	**0.31±0.15**^{3}
Corona width	−0.17±0.19	−0.01±0.10	0.13±0.09	0.08±0.07	0.39±0.25	0.20±0.13

$^1P<0.01$, $^2P<0.001$, $^3P<0.05$.

homogeneity of variance. We examined the correlation between male and female reproductive success using Spearman's rank correlation and λ_j and λ_k for each individual. Individuals that did not produce offspring through either male or female function were not included in this analysis.

Results

SELECTION THROUGH FEMALE FUNCTION

Patterns of selection among populations through female function were generally congruent. Significant selection differentials, which measure the combined effects of direct and indirect selection on a trait, were evident in three of the six populations investigated. We identified significant directional selection for more flowers in three of the four populations in which there was significant variation in flower number (Table 3). In addition, we identified directional selection for taller plants ($s = 0.29 \pm 0.06$) in population 208 as well as directional selection for larger flowers ($s = 0.31 \pm 0.15$) in population 254.

Multivariate analysis identified positive linear selection gradients for flower number in all four populations that had variation in this trait (Table 4), although the linear selection gradient was not significant in population 204. Directional selection for taller plants was also detected in population 208 ($\beta' = 0.19 \pm 0.07$). As none of the quadratic regression coefficients were significant, for ANCOVA we did not include the quadratic components from the model. In addition, none of the interactions between population and the traits were significant and were removed from the ANCOVA model. ANCOVA revealed a significant effect of flower number on seed set for the four populations with variation in flower number ($F_{1,398} = 44.44$, $P < 0.001$; Table 5A). A likelihood-ratio test indicated that seed set differed among these populations ($G_1 = 47.80$, $P < 0.001$). Among the six populations, ANCOVA revealed positive selection for

Table 4. **Standardized selection gradients through female function in six populations of *Narcissus triandrus*. Linear (β') and quadratic (γ') regression coefficients and their standard errors are shown. We calculated the selection differentials using relative fitness (w), and tested the statistical significance using a log ($w + 1$) transformation to improve normality of the residuals. Selection gradients were calculated using standardized traits. Effects in bold are significant at $P < 0.05$.**

Trait	Population											
	P153		P204		P207		P208		P245		P254	
	β'	γ'	β'	γ'	β'	γ'	β'	γ'	β'	γ'	β'	γ'
Flower number	—	—	0.19 ±0.12	0.10 ±0.07	**0.29**^{1} **±0.09**^{2}	−0.03 ±0.09	**0.27** **±0.07**^{2}	0.06 ±0.06	—	—	**0.62** **±0.16**^{3}	0.001 ±0.09
Height	0.13 ±0.26	−0.18 ±0.15	−0.11 ±0.05	−0.15 ±0.07	0.02 ±0.10	0.04 ±0.08	**0.19** **±0.07**^{1}	0.02 ±0.05	0.57 ±0.27	0.51 ±0.20	0.13 ±0.16	0.20 ±0.11
Flower length	−0.06 ±0.25	−0.02 ±0.13	0.02 ±0.12	0.10 ±0.11	−0.04 ±0.11	−0.08 ±0.06	0.06 ±0.06	−0.04 ±0.04	0.15 ±0.25	0.06 ±0.17	0.12 ±0.15	−0.04 ±0.06
Corona width	−0.14 ±.24	−0.25 ±0.16	−0.02 ±0.10	−0.01 ±0.06	0.07 ±0.11	0.09 ±0.06	0.02 ±0.06	−0.08 ±0.04	0.13 ±0.27	−0.22 ±0.17	0.01 ±0.13	0.01 ±0.03

$^1P<0.05$, $^2P<0.01$, $^3P<0.001$.

Table 5. Analysis of covariance of the relation between standardized trait values and seed set among populations of *Narcissus triandrus* using a mixed model analyses. (A) The four populations of *N. triandrus*, which had variation in flower number. (B) Six populations of *N. triandrus*. We tested population, a random effect, using a likelihood ratio test. All interactions were nonsignificant and removed from the model in a stepwise manner. Therefore, the final analysis included only traits and population as main effects. We estimated statistical significance using a square root (seed set + 1) transformation to improve normality of the residuals. Effects in bold are significant at $P<0.05$.

(A) Effect	Statistic	*P*
Flower number	**$F_{1,398}$=44.44**	**<0.001**
Height	$F_{1,399}$=1.13	0.29
Flower length	$F_{1,399}$=1.14	0.29
Corona width	$F_{1,398}$=0.03	0.85
Population	**G_1=47.8**	**<0.001**

(B) Effect	Statistic	*P*
Height	**$F_{1,542}$=9.75**	**<0.01**
Flower length	$F_{1,542}$=0.37	0.54
Corona width	$F_{1,542}$=0.36	0.55
Population	**G_1=185.2**	**<0.001**

taller plants ($F_{1,542} = 9.75$, $P < 0.01$; Table 5B), when flower number was removed from the analysis. A likelihood-ratio test indicated that seed set also differed among populations ($G_1 = 185.2$, $P < 0.001$).

SELECTION THROUGH MALE FUNCTION

In both of the populations that we investigated selection through male function, interplant distance had a large influence on male reproductive success (population 204 $\gamma_d = -0.75$; population 254 $\gamma_d = -0.43$; Table 6). Negative values of γ_d indicate declining pollen dispersal with distance between mates with individuals siring a high proportion of their neighbors' seed. Selection through male function was also detected in both populations. The specific patterns of selection were not qualitatively affected by the inclusion of distance so we only present the results of models that incorporated distance. The univariate selection gradients revealed weak directional selection for fewer flowers in population 204 ($s = -0.52$) and for larger flowers in 254 ($s = 0.35$). None of the other univariate selection gradients was significant (Table 6). However, several multivariate selection gradients were significant (Table 7). In population 204 we found weak directional selection for fewer flowers ($\beta' = -0.55$) and a significant negative quadratic regression coefficient for corona width ($\gamma' = -0.35$). In population 254, a positive selection gradient for flower length ($\beta' = 0.34$) and a significant negative quadratic coefficient for flower length ($\gamma' = -0.46$) were evident. Negative quadratic coefficients can indicate stabilizing selection. We also found a significant positive quadratic regression coefficient for flower number ($\gamma' = 0.14$) in population 254, which can indicate disruptive selection.

Table 6. Univariate selection gradients for male function in two populations of *Narcissus triandrus*. We determined the significance using the chi-square distribution and pedigree simulations after Morgan and Conner (2001), respectively (based on 1000 bootstraps). Effects in bold are significant at $P<0.05$.

Trait	P204	*LLR*	P254	*LLR*
Height	−0.23	$3.19^{\mathrm{ns,ns}}$	0.03	$0.11^{\mathrm{ns,ns}}$
Flower number	**−0.52**	**6.14^1**	0.12	$1.03^{\mathrm{ns,ns}}$
Flower length	−0.03	$0.08^{\mathrm{ns,ns}}$	**0.35**	**6.01^1**
Corona width	−0.12	$0.78^{\mathrm{ns,ns}}$	0.002	$0.00^{\mathrm{ns,ns}}$
Distance	**−0.75**	**42.73^2**	**−0.43**	**26.18^2**

$^1P<0.05$, $^2P<0.001$.

In cases in which we detected significant quadratic regression coefficients we used a modification of the method of Mitchell-Olds and Shaw (1987) to determine if this represented stabilizing or disruptive selection. First, we determined whether the fitness maximum or minimum of a trait (z_i) was within the observed range of phenotypic values by taking the derivative of w with respect to z_i and setting the fitness equation to 0 (see also Simms and Rausher 1989; Simms 1990). For the log-linear equation the maximum or minimum is $z^* = -\beta/2\gamma$. Second, we tested the significance of the maximum or minimum using a bootstrap analysis by randomly resampling progeny with replacement and recalculating both the selection gradients and z^* to construct 95% confidence intervals for z^* using the percentile method. As we had to rerun PatQuest manually each time with a resampled progeny dataset we conducted 100 bootstraps.

We found that the fitness maximum for corona width in population 204 ($z^* = 0.13$, lower CI $= -1.57$, upper CI $= 0.90$) was within the range of phenotypic values (−2.04 to 3.00). In population 254, the maximum for flower length ($z^* = 0.08$, lower CI $= -1.28$, upper CI $= 1.29$) was also within the range of phenotypic values (−4.60 to 1.89). This indicates that both traits are under stabilizing selection. The fitness minimum for flower number ($z^* = -0.18$, lower CI $= -0.20$, upper CI $= 3.63$) was also within the range of phenotypic values (−0.94 to 4.25), which may indicate disruptive selection. However, stabilizing or disruptive selection may be mistakenly identified using this method if selection differentials are weak and the instantaneous slopes to one side of the maximum or minimum are very small. In this situation the phenotypic variation is virtually neutral over a part of its range. Consequently, we plotted the relation between male reproductive success and each of the traits with significant nonlinear selection gradients (online Supplementary materials Figure S1). The fitness function for flower number is flat to the left of the fitness

Table 7. Multivariate selection gradients through male function in two populations of *Narcissus triandrus*. We determined the significance using the chi-square distribution and pedigree simulations after Morgan and Conner (2001), respectively (500 bootstraps). Effects in bold are significant at $P < 0.05$. Population 204, full model: $\beta = 27.37$, df = 9, $P_{(chi)} < 0.001$; distance $\gamma_d = -0.76$, $LLR = 43.79$, $P_{(chi)} < 0.001$, $P_{(sim)} < 0.001$. Population 254 full model: $\beta = 46.41$, df = 9, $P_{(chi)} < 0.001$; distance $\gamma_d = -0.42$, $LLR = 23.53$, $P_{(chi)} < 0.001$, $P_{(sim)} = 0.40$.

Trait	Population 204				Population 254			
	β'	*LLR*	γ'	*LLR*	β'	*LLR*	γ'	*LLR*
Flower number	**−0.55**	**5.12**^{1}	−0.11	0.013	0.05	0.17	**0.14**	**6.754**^{2}
Height	0.04	0.05	0.07	0.32	0.045	0.11	−0.08	1.31
Flower length	0.002	0.0004	0.08	1.06	**0.34**	**5.25**^{1}	**−0.46**	**6.744**^{2}
Corona width	0.099	0.58	**−0.35**	**7.21**^{2}	−0.03	0.05	0.16	4.24^{1,3}

$^{1}P<0.05$, $^{2}P<0.01$, $^{3}P<0.1$.

minimum, suggesting directional selection for larger floral displays in population 204. The graphs of flower length and corona width demonstrate a clear decline in fitness on both sides of the maximum thus confirming stabilizing selection.

VARIATION IN FEMALE AND MALE REPRODUCTIVE SUCCESS

Male reproductive success deviated significantly from a uniform distribution, although only the bootstrap analysis was significant in population 254 (population 204 LLR = 250.48, df = 135 $P_{(chi)} < 0.001$, $P_{(sim)} < 0.001$; population 254 LLR = 99.28, df = 112 $P_{(chi)} > 0.05$, $P_{(sim)} < 0.001$; Fig. 1A,B). Female reproductive success also varied in both populations (Fig. 1C,D). In population 254, variance in female (3.24×10^{-4}) and male reproductive success (3.26×10^{-4}) did not differ significantly ($F_{1,201} = 0.05$, $P = 0.83$). However, in population 204, variance in male reproductive success (2.7×10^{-4}) was significantly higher than variance in female reproductive success (7.7×10^{-5}) ($F_{1,255} = 6.66$, $P < 0.01$). There was a significant negative correlation between male and female reproductive success in population 254 (Spearman's $\rho = -0.29$, $N = 73$, $P < 0.01$; Fig. 2A). The same negative trend was also evident in population 204 although the relation was not significant (Spearman's $\rho = -0.14$, $N = 91$, $P = 0.17$; Fig. 2B).

Discussion

The ecology and genetics of heterostylous populations have been investigated extensively since the pioneering studies of Darwin

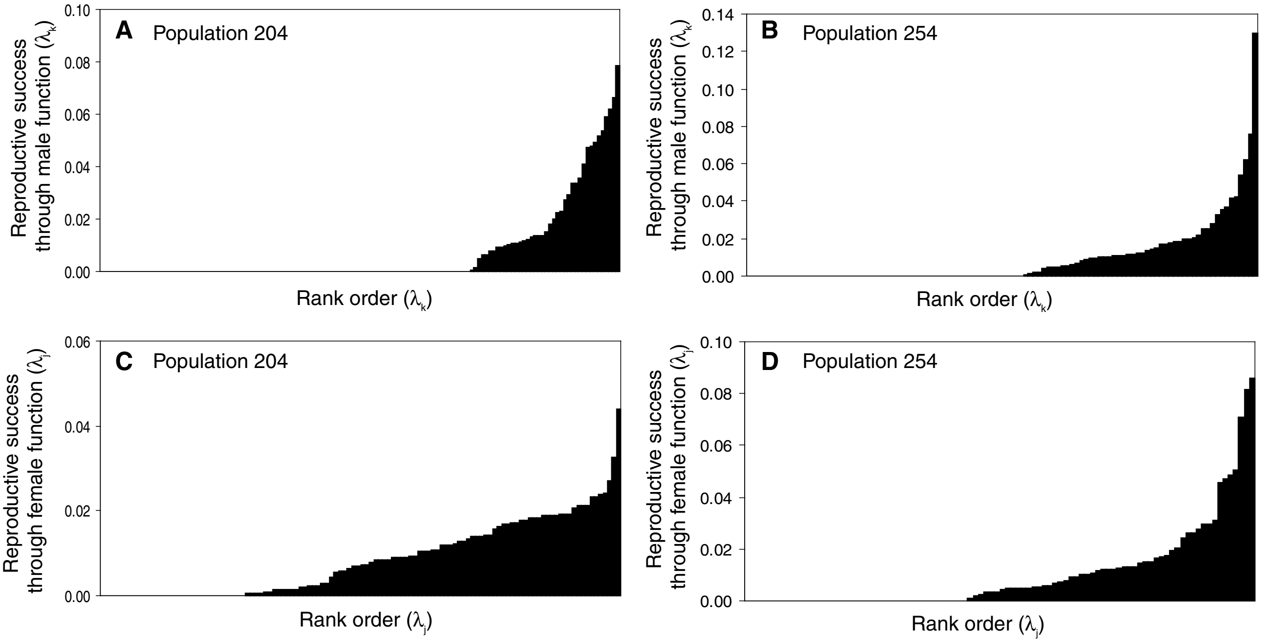

Figure 1. Male and female reproductive success (λ) in two populations of *Narcissus triandrus*. Variation in male reproductive success in population 204 (A) and population 254 (B) and variation in female reproductive success in population 204 (C) and population 254 (D). We used seed number as a measure of female fitness and male fitness was determined using parental and offspring multilocus genotypes and estimated using PatQuest. λ-values are sorted in increasing order.

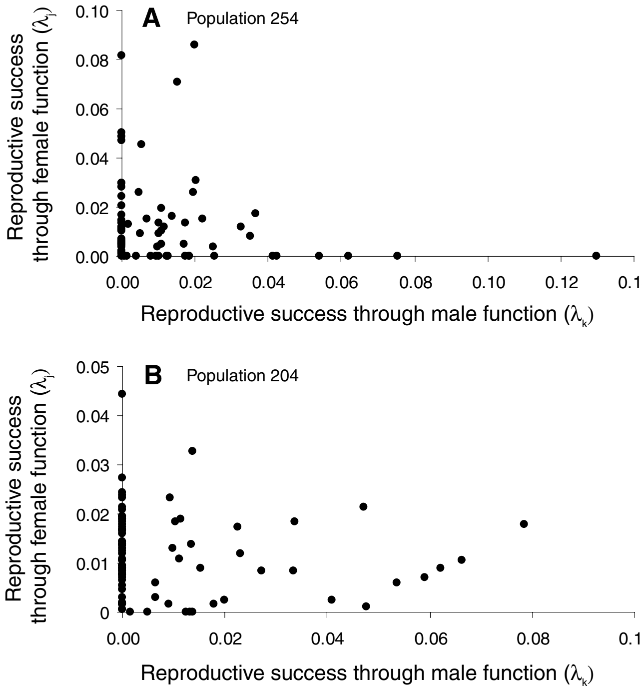

Figure 2. Correlation between male and female reproductive success (λ) in two populations of *Narcissus triandrus*. Correlation between male and female reproductive success in (A) population 254, Spearman's $\rho = -0.29$, $N = 73$, $P < 0.01$, and (B) population 204, Spearman's $\rho = -0.14$, $N = 91$, $P = 0.17$.

(1877). These floral polymorphisms are maintained in populations by negative frequency-dependent selection and function to promote proficient animal-mediated cross-pollination. Despite the extensive literature on heterostyly (reviewed in Barrett 1992) there have been no investigations of natural selection on floral traits (although see Nishihiro et al. 2000). Our study of the patterns of phenotypic selection in natural populations of tristylous *N. triandrus* provides several novel insights into the reproductive biology of animal-pollinated plants. Below we summarize our findings and their general implications for floral function and evolution.

We found evidence of a negative relation between male and female function in population 254 of *N. triandrus*. To our knowledge, this represents the first example of a trade-off between male and female reproductive success detected in a natural population of a hermaphroditic plant. We also found significantly greater variance in male than female reproductive success in population 209, a pattern predicted by Bateman's principle (1948). However, variance in male and female reproductive success was similar in population 254 indicating that ecological context (i.e., variation in pollination environment or resources among populations) has the potential to affect patterns of sex-specific selection. We detected significant directional selection through female function for increased flower number and for taller plants, as would be expected if local resources limit growth and seed set. Although the strongest determinant of male reproductive success was intermate distance, we detected selection on floral design through male function, but not female function. Specifically, we found evidence for stabilizing selection for corona width in population 204 and for flower length in population 254. Our results indicate that in populations of *N. triandrus* selection on morphological traits that promote effective pollen dispersal is likely to be stronger through male function than through female function.

SELECTION THROUGH FEMALE FUNCTION

Plant size is a common determinant of female fertility in many plant species with larger more vigorous plants producing more seeds (Harper 1977; Herrera 1993; Conner and Rush 1997; Benitez-Vieyra et al. 2006). Fitness gain through female function in animal-pollinated plants is predicted to initially increase with pollinator visitation but to plateau as pollen receipt reaches a level necessary for maximum seed set. Consequently, when pollinators are plentiful and resources are limited there should be weak selection on attractive traits through female function. Our study of variation in female reproductive success identified a strong influence of flower number on female fitness (Tables 3–5). In addition, when flower number was removed from the model, we found a significant influence of height on total seed production for all six populations (Table 5B). Flower number and height are likely correlated with overall resource status and possibly age. Heterogeneity in local resources probably contributes to much of the variation in female reproductive success among individuals in populations of *N. triandrus*.

We found no compelling evidence of selection through female function for traits related to floral design in *N. triandrus*. Weak or no selection through female function on floral traits has also been reported in other plant species, particularly under resource-limited conditions (see Ashman and Morgan 2004 for a review). However, we identified significant selection for flower length in population 254 (Table 3). Interestingly, this particular population was previously identified as one of only two of 11 populations of *N. triandrus* exhibiting pollen limitation of seed set (Hodgins and Barrett 2006). With this exception, the absence of selection through female function on traits involved in floral design is likely due to the generally low levels of pollen limitation experienced by most populations of *N. triandrus* (Hodgins and Barrett 2006). Consequently, selection on floral traits, such as flower size or shape, is more likely to result from variation among plants in male reproductive success.

DETERMINANTS OF MALE REPRODUCTIVE SUCCESS

Several researchers have identified considerable near-neighbor mating in animal-pollinated species using genetic markers (e.g., Meagher 1991; Devlin et al. 1992; Smouse et al. 1999; Wright and Meagher 2004). Similar distance effects have also been detected in wind-pollinated species (e.g., Vassiliadis et al. 2002; Bacles et al. 2005; Burczyk et al. 2006). Although spatial scale can influence dispersal parameter estimates, we were able to detect strong effects of interplant distance on reproductive success in *N. triandrus*, despite the limited area occupied by our study populations (population 204 = 553 m^2; population 254 = 371 m^2). Our results revealed that mating generally occurs among near-neighbors in populations of *N. triandrus*. For example, our distance estimates indicate that 89% and 64% of mating occurred among individuals separated by less than 5 m in population 204 and 254, respectively. However, long-distance gene flow from unsampled pollen donors is also a common phenomenon in studies of natural populations and leads to underestimates of the dispersal parameter (Morgan and Conner 2001). Exclusion analyses revealed that approximately 29% of progeny in both populations may have been sired by individuals outside our study areas, and this likely influenced the accuracy of our dispersal estimates. However, exclusion analysis does not account for genotyping error and 29% is likely an overestimate of the number of uncensused pollen donors.

We found evidence for stabilizing selection through male function for corona width in population 204 and flower length in population 254. Both traits are positively correlated with flower size in *N. triandrus*. Stabilizing selection on flower size is difficult to explain based on pollinator attraction, because larger flowers should increase pollinator visitation and consequently pollen export and siring success. Cresswell (1998) suggested several possible mechanisms that might underlie stabilizing selection on floral traits, including the structural match between pollinators and flowers and trade-offs in resource allocation among traits. Segregation of pollen on a pollinator's body is important for effective cross-pollen transfer in heterostylous species (reviewed in Ganders 1979; Lloyd and Webb 1992). In *N. triandrus*, variation in flower size influences the positional relations of the sex organs and may disrupt pollen transfer, especially among mid-level organs contributing to the loss of the M-morph from populations (Barrett et al. 2004). Therefore, longer or wider flowers may result in less precise pollen transfer and gamete wastage resulting in stabilizing selection for relatively uniform floral architecture.

Flower size is generally considered to represent an attractive trait to pollinators (Ashman and Morgan 2004). However, in *N. triandrus* we found no evidence for positive selection through male function on flower-size related traits. This result, although contrary to theoretical predictions for selection on attractive traits based on the "male function hypothesis" (reviewed in Burd and Callahan 2000), is consistent with recent work using marker-based paternity analysis (Meagher 1991; Smouse et al. 1999; Elle and Meagher 2000; Morgan and Conner 2001; van Kleunen and Ritland 2004). For example, Meagher (1991) and Smouse et al. (1999) found no evidence for selection on floral characters in natural populations of *Chamaelirium luteum* and Elle and Meagher (2000) found significant positive selection for flower size in only one of three experimental populations of *Solanum carolinense*. Although statistical power can be limited in marker-based studies (Morgan and Conner 2001) and may contribute to this pattern, collectively these results indicate that patterns of selection for flower size may be more complex than predictions made from pollinator visitation rates alone.

Marker-based studies of male selection gradients rarely investigate stabilizing selection, despite the prediction that some floral traits are likely to be functionally optimized for interactions with pollinators. The few studies that have examined this form of selection have generally found evidence for stabilizing selection. For example, Morgan and Conner (2001) reported stabilizing selection on anther exertion and flower size in *Raphanus raphanistrum* and Wright and Meagher (2004) also found evidence for stabilizing selection on calyx diameter in *Silene latifolia*. Although further studies on a wider range of taxa are clearly desirable, these results in conjunction with our data suggest that stabilizing selection on floral characters may be an important feature of mating success in animal-pollinated populations.

The nature of selection on display size through male function differed between the two populations of *N. triandrus* that we investigated. In population 254, we identified directional selection for more flowers, which is predicted based on the advantage of larger floral displays in attracting pollinators. In contrast, in population 204 there was no evidence of selection for larger display sizes but instead selection in the opposite direction. Although larger displays are predicted to increase male fitness, flower number is not always correlated with male success (Meagher 1991; Elle and Meagher 2000). Interflower self-pollination (geitonogamous pollination) resulting in pollen discounting (i.e., reduced outcrossed siring success due to self pollination—Harder and Barrett 1995) could occur in *N. triandrus* and could offset any numerical advantage to male function that increased flower number provides.

COMPARISON OF MALE AND FEMALE REPRODUCTIVE SUCCESS

Variation among individuals in male reproductive success should exceed variation among individuals in female reproductive success because of the larger number of gametes produced by males compared to females (Bateman 1948). In population 204 this prediction was supported indicating that there is greater opportunity for selection through male than female function in this population (Fig. 1A,C). However, in population 254 there was no evidence for sex-specific differences in variance (Fig. 1B,D). This difference between the two populations is a result of higher variation in female fitness in population 254 compared to population 204. In population 254, 56% of plants did not set seed probably because of limited pollinator service and/or low resource availability. In the same year we detected lower seed set of open-pollinated compared to pollen-supplemented flowers in this population implicating pollen limitation of female fertility (Hodgins and Barrett 2006). In contrast, in population 204 only 18% of plants failed to produce seed and a study conducted the following year at this site did not detect pollen limitation. Regardless of the cause(s) of variation in female fertility, this finding indicates that that reproductive environment is potentially an important factor in determining the strength of sex-specific selection on floral traits (and see Fishman and Willis 2008).

Our results are in accord with a growing body of literature questioning the generality of the "male function" hypothesis (Wilson et al. 1994; Ashman and Morgan 2004). Studies comparing variation in male and female reproductive success using genetic markers have provided mixed results. Several report more variable male success compared to female success (Meagher 1986; Wright and Meagher 2004), whereas others have found the opposite pattern (Devlin and Ellstrand 1990; Morgan and Schoen 1997; Caruso et al. 2005). Sex-specific variation in reproductive success can also vary between years in the same population (Conner et al. 1996). However, it is important to note that maximum likelihood estimates of male fertility are derived from statistical models. In contrast, female fertility is measured from direct counts of the number of seeds produced per plant. As a result, the estimates of variation in male fertility may commonly be underestimated (Smouse and Meagher 1994). Unfortunately, we cannot assess to what extent this potential bias may have influenced our ability to detect sex-specific differences in reproductive success in population 254.

Several studies of hermaphroditic plants have found weak positive correlations between male and female reproductive success. This pattern is predicted when increased resources improve the fitness of both sex functions (Broyles and Wyatt 1990; Devlin and Ellstrand 1990; Conner et al. 1996). However, for those individuals of *N. triandrus* that successfully reproduced, we identified a negative correlation between male and female function in one population and a negative trend in the other. Elle and Meagher's (2000) study of experimental arrays of *S. carolinense* also found a negative correlation between male and female reproductive success. These results suggest the potential for differences in functional gender among individuals (Lloyd 1980). In population 254, we found that many plants that failed to set seed had been visited by pollinators, as they sired seeds. Resource limitation likely explains some of the failure in seed set, although variation in morphology may also contribute to the pattern, with some individuals better able to export pollen and others better able to receive pollen. In particular, we found evidence that large-flowered individuals had higher seed set, whereas flowers of intermediate size had the greatest reproductive success through male function.

Variation in functional gender in heterostylous plants can have important consequences for sexual-system evolution. For example, differences in the functional gender of morphs can lead to the evolution of dioecy (Lloyd 1979; reviewed in Casper 1992). However, in the population in which we detected a significant relation (population 254) both the L- and the S-morphs exhibited a negative correlation between male and female reproductive success (L-morph Spearman's $\rho = -0.25$; S-morph Spearman's $\rho = -0.60$). This indicates that the negative correlation we detected is not associated with morph-specific differences in gender. However, to our knowledge, no other study has demonstrated a trade-off between male and female function in a heterostylous population, despite considerable interest in the potential for gender specialization (reviewed in Casper 1992).

Our study has revealed divergent patterns of natural selection through male and female function in *N. triandrus*. The occurrence of stabilizing selection and the different patterns of selection through male and female function demonstrate the importance of assessing nonlinear selection gradients and quantifying selection through both sex functions. We identified selection on floral display size through female function and selection on components of flower size through male function. Directional selection for more flowers and larger plants through female function may indicate that resources generally limit seed set in this species. However, although we found evidence for greater variation in male fitness in one population this did not generally translate into positive selection for attractive traits through male function, as might have been predicted by hypotheses concerning sex-specific selection on attractive traits. Consequently, in wild populations of *N. triandrus* pollinator attraction alone does not appear to drive selection on floral traits, at least in the years and sites that we studied. In contrast, evidence for stabilizing selection on floral traits through male function suggests that floral design may be of greater significance for male fitness because of its effects in promoting cross-pollination among the floral morphs of *N. triandrus*.

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Supplementary Material

The following supplementary material is available for this article:

Figure S1. The predicted relation between relative fitness through male function and the standardized trait values for three traits where significant nonlinear components were found in two populations of *Narcissus triandrus*.

This material is available as part of the online article from:
http://www.blackwell-synergy.com/doi/abs/10.1111/j.1558-5646.2008.00404.x
(This link will take you to the article abstract).