**Is variation in flower shape and length among native and non-native populations of
Nicotiana glauca a product of pollinator-mediated selection?**

Macarena García¹, Santiago Benítez-Vieyra¹, Alicia Noemí Sérsic¹, Anton Pauw², Andrea
Arístides Cocucci¹, Anna Traveset³, Federico Sazatornil¹ and Valeria Paiaro^{1*}

1- Instituto Multidisciplinario de Biología Vegetal (IMBIVCONICET), Universidad Nacional
de Córdoba, Av. Vélez Sársfield 1611, C.C. 495, 5000 Córdoba, Argentina

2- Department of Botany and Zoology, Stellenbosch University, Private Bag X1, Matieland
7602, South Africa

3- Instituto Mediterráneo de Estudios Avanzados (CSICUIB), C/ Miquel Marquès, 21 - 07190
Esporles, Mallorca, Balearic Islands, Spain

*corresponding author: e-mail: vpaiaro@gmail.com

Running title: Flower shape and length in native and non-native pollination environments

Keywords: geometric morphometrics, hummingbirds, phenotypic selection, self-pollination,
sunbirds

Total word count: 6379

Number of figures: 7

Number of tables: 3

Appendices: 1

Abstract

Due to drastic changes in pollinators between native and invaded habitats, we might expect that pollinator-mediated selection on floral traits of alien plants differ from that in their native ranges. Here, through geometric morphometric tools and phenotypic selection analyses, we examined whether adaptation in flower shape and length occurred in *Nicotiana glauca* as a response to pollinator selection in contrasting pollination environments. We assessed populations of this plant species in the native range (South America), where plants depend on hummingbird pollination, and in two invaded areas, one where sunbirds act as pollinators (South Africa), and another where nectar feeding birds are absent and reproduction is entirely by autonomous self-pollination (Mallorca, Spain). Corolla length and shape varied significantly among pollination environments. Non-native sites were less variable and their range of variation fell within the native range of variation. Flower length in native populations and in a South African population matched the bill length of their respective pollinators. In contrast with the straight floral tubes in the native range, both non-native areas had significantly curved tubes. Curvature may improve the fit with the curved bills of sunbirds in South Africa (versus straight beaks of hummingbirds) and may enhance self-pollination in Mallorca, but this similarity between invaded areas may equally be due to drift and a shared colonization route. We found spatial variation in selection acting on corolla length but not on corolla shape. Overall, selection patterns were not consistent with floral trait variation. Although some results are consistent with both drift and selection, our study suggests that population divergence in flower shape and length is more likely the result of long-term diversifying pollinator-driven selection, which is difficult to detect by studying a single selection event.

47 **Introduction**

48 Species introduction into new environments generally involves drastic changes in the selective
49 regime, which can lead to adaptive evolution (Sakai et al 2001; Murren et al 2009; Molina-
50 Montenegro et al 2011; Westley 2011; Colautti and Lau 2015). Simultaneously, the reduction in
51 population size during the colonization event will favour genetic drift and will reduce genetic
52 diversity of introduced plant populations, limiting the possibility of adaptive evolution of traits
53 under new ecological conditions (Charlesworth 2003; Dlugosch and Parker 2008; Charlesworth et
54 al 2009; Colautti et al 2010). The success of invasive species may to some extent be determined by
55 their capability to adapt to new environments (Colautti and Lau 2015).

56 Pollinators, which are one of the main agents of selection on floral traits (Caruso et al 2019),
57 may differ substantially in their abundance and identity between native and non-native habitats
58 (Traveset and Richardson 2014; Jiménez-Lobato et al 2018; Issaly et al. 2020). Alien animal-
59 pollinated plants, even those highly specialized in their native range, may respond by adapting to
60 functionally similar or completely novel pollinators in non-native areas (Richardson et al 2000;
61 Geerts and Pauw 2009; Traveset and Richardson 2014). They may also change their mating system
62 or rely on some kind of asexual reproduction if suitable pollinators are lacking in the new
63 environment (Richardson et al 2000; Ollerton et al 2012; Traveset and Richardson 2014; Jiménez-
64 Lobato et al 2018; Issaly et al. 2020). Phenotypic selection studies have provided a valuable
65 snapshot of spatial variation in selection on floral traits across populations with different pollinator
66 faunas (e.g., Nuñez-Farfan and Schlichting 2005; Kingsolver and Pfennig 2007; Gómez et al 2008).
67 Geographic variation in pollinators, the so-called pollination climate, may result in concomitant
68 spatial variation in the nature of selection affecting floral traits, especially if pollinators differ in

morphology, foraging behavior or flower trait preferences (Gómez and Zamora 2000; Herrera et al 2006; Moeller 2006; Gómez et al 2008; Nattero et al 2010a,b).

Selection in new habitats may occur not only in response to the presence of new pollinators but also in response to their absence (Hierro et al 2005). When pollinators are scarce or absent in the new range, selection would favor characters that enhance opportunities for selfing in order to ensure reproduction (Fenster and Ritland 1994; Moeller 2006). Morphological changes that are associated with selfing include reduced herkogamy (Takebayashi et al 2006; Bodbyl-Roels and Kelly 2011), bent or severely crumpled corolla tubes (Schueller 2004) and a reduction in flower size, which can be the result of differential selection on resource allocation to attractiveness function (Lloyd 1987; Charlesworth and Morgan 1991; Fishman and Willis 2008).

Our understanding of how invasive plants can adapt to changes in pollinators is limited. This is an important gap in our knowledge because pollinator environments have drastic implications for plant reproduction and fecundity, and in turn for long-term evolution of invasive species. However, very few studies quantified selection on floral traits in both native and non-native populations (but see Murren et al 2009; Jiménez-Lobato et al 2018). These kinds of studies are crucial to identify traits under selection in invaded environments and to determine how selection may differ between invaded and native ranges, which, in turn, may be important to determine invasion rate and impacts on native ecosystems (Colautti and Lau 2015).

Nicotiana glauca R. Graham (Solanaceae) is an ornithophilous South American shrub (Goodspeed 1954) that successfully invaded semi-arid regions around the world with different pollinator environments (Ollerton et al 2012; Issaly et al 2020). Here, we studied floral shape and length and their phenotypic selection in the native range, where plants depend on hummingbird-pollination, and in two invaded areas, one where sunbirds act as pollinators, South Africa, and

92 another where nectar feeding birds are absent and reproduction is entirely by autonomous self-
93 pollination, the Balearic island of Mallorca (Issaly et al 2020). Previous studies on *N. glauca* found
94 high variation in corolla length associated with bill length of hummingbird pollinators across the
95 native range (Nattero et al 2011) and among non-native populations in the USA (Schueller 2007).
96 In addition, another study showed that pollinator-mediated phenotypic selection potentially shaped
97 corolla length in several native populations (Nattero et al 2010a). Finally, a recent study in this
98 species found that increased self-pollination ability in Mallorca, a non-native environment without
99 pollinators, was not associated with a reduction in anther-stigma distance (Issaly et al 2020).
100 Instead, this study proposed that a narrow and curved floral tube could favor pollen deposition on
101 the stigma when the corolla and stamens detach from the gynoecium, bringing about delayed self-
102 pollination (*sensu* Lloyd and Schoen 1992) in this invaded area. Differences in pollinator
103 morphology in different areas may also be a factor that mediates selection on flower shape of *N.*
104 *glauca* (Gómez et al 2008; Benítez-Vieyra et al 2009). Birds that hover while feeding, including
105 most hummingbirds, generally have straight bills, whereas birds that perch while feeding, such as
106 the Old World sunbirds, have bills that are decurved to some extent (Paton and Collins 1989),
107 because they probe in an arc motion from a fixed perching position (Westerkamp 1990; Temeles
108 et al 2009).

109 We hypothesized that changes in flower shape and length of *N. glauca* occur in native and
110 non-native ranges as a response to selection by the different pollination environments (i.e., native
111 hummingbird-pollination, non-native sunbird-pollination, and non-native without pollinators).
112 Specifically, we expected to find significant levels of phenotypic differentiation in corolla length
113 and shape across pollination environments. In relation to flower size, we expected the smallest (i.e.
114 shortest and narrowest) corolla tubes in non-native areas without pollinators, and differences in

flower length between populations whose main pollinator varies in bill length both in the native and South African ranges. Regarding flower shape, we expected to find straight corollas in the native range and curved corollas in both non-native pollination environments. Finally, we expected that patterns of pollinator-mediated phenotypic selection would be consistent with floral trait differentiation across populations.

Material and Methods

Study species and sites

Nicotiana glauca is a fast-growing shrubby tree native to central Argentina and Southern Bolivia (Goodspeed 1954). It has been widely introduced, either deliberately as ornamental, or accidentally, and has become a weed in the subtropical regions of four continents (Cronk and Fuller 1995). The species is listed in the Global Invasive Species Database (<http://www.issg.org/ase/welcome/>) and is considered invasive in regions such as Europe and South Africa.

Almost all year round, in both the native and non-native range, plants of *N. glauca* produce many inflorescences that bear numerous flowers, which open throughout the day and last about 3–4 days. Flowers have long (30–57 mm) and wide (6–11 mm) tubular corollas with a reduced corolla limb (Nattero et al 2011). The corolla is greenish-yellow when young, changing to bright yellow when aging. Some populations in Argentina have colour polymorphism including dark red, reddish yellow and yellow morphs (ANS, AAC and VP, pers. obs.). Anthers reach the corolla tube opening and the stigma may be as high as or a little higher than the anthers (Issaly et al 2020). Flowers are scentless and produce large amounts (ca. 20 μ L) of low concentration (ca. 25% sucrose) nectar

(Galetto and Bernardello 1993). The two-valved dehiscent capsules contain hundreds of tiny seeds.

Propagation may also occur clonally.

In its native range, *N. glauca* is visited by different hummingbird species assemblages depending on the geographic location of the populations (Nattero and Cocucci 2007; Nattero et al 2010a, 2011). Outside the Americas the species interacts with different bird pollinators, such as sunbirds in South Africa, or set seeds exclusively by selfing in areas where nectar feeding birds are absent, such as the Mediterranean Basin (Geerts and Pauw 2009; Ollerton et al 2012; Issaly et al 2020). Despite their typical perching behavior, Old World sunbirds, especially the malachite sunbird, *Nectarinia famosa*, must often hover to feed from the flowers of *N. glauca* (Geerts and Pauw 2009). The species is self-compatible throughout the native and non-native range, although in pollinator poor areas plants have a much higher capability for autonomous self-pollination than elsewhere (Issaly et al 2020). Conversely, pollination by hummingbirds and sunbirds increases reproductive success of plants both in native and in South African areas, respectively (Geerts and Pauw 2009; Issaly et al 2020).

To compare the different pollination environments of *N. glauca*, we studied three native populations, two in Argentina and another in south Bolivia, two non-native populations in South Africa, and two non-native populations on the island of Mallorca (Balearic Islands, Spain; Table 1). The first records of *N. glauca* were in 1884 for South Africa (Bromilow 2001) and between 1827 and 1879 for the Balearic Islands (Moragues and Rita 2005). Populations within each region were separated by at least 2 km. Study populations were located either in semiarid naturally disturbed (riverbanks or alluvial sediments) or artificially disturbed areas (roadsides, farmlands and abandoned quarries). We carried out samplings during spring and summer (in both hemispheres) between 2012 and 2014.

Pollinator assemblages

To determine pollinator assemblages in the study populations, we complemented previous detailed studies (Geerts and Pauw 2009; Nattero et al 2011; Ollerton et al 2012) with additional observations. We performed observations with close focusing 8×40 binoculars during 10-15 periods of 20 min from 7:00 to 14:00 h on two sunny days per population. We also surveyed the two Mallorcan populations for nocturnal visitors from 19:00 to 22:00 h on one evening each. Pollinator visitation rate analyses for the study populations were published elsewhere (Issaly et al 2020), and here we only present a summary of pollinator composition and relative visitation frequencies (Table 2). We took into account only legitimate visits (i.e. those that touched floral fertile parts), and included both hovering and perching visits in the case of sunbirds. For each observed hummingbird and sunbird species, we obtained mean bill length (i.e., exposed culmen taken from the nostril to the end of the bill) in the respective geographic provinces of the study sites, from Nattero et al (2011) and from the South African Bird Ringing unit (SAFRING) database, respectively.

Floral traits

We labeled between 43 and 100 plants in each population (Table 1) and collected three flowers per plant. We photographed flowers laterally with a reference scale using a Nikon Coolpix 5400 camera, following a standardized protocol, with the camera's axis perpendicular to the corolla tube surface. We quantified corolla tube shape by means of geometric morphometrics (Bookstein 1997; Zelditch et al 2012). For each flower of *N. glauca*, 18 coplanar landmarks along the corolla tube were defined (Fig. 1). The landmarks 1, 3, 8-11, 16 and 18 represent the limits between different

kinds of tissues or anatomical structures (i.e., corolla limb-corolla tube, corolla tube-calyx, and calyx-pedicel). The landmarks 2, 6, 7, 12, 13 and 17 were points of minimum or maximum curvature. The remaining four landmarks (4, 5, 14 and 15) were semilandmarks, a series of locations to capture the homologous curved outlines of the corolla-tube between the remaining landmarks (Gunz and Mitteroecker 2013). We obtained landmark configurations from each individual using the program TpsDig ver. 2.16 (Rohlf 2010). We performed a generalized Procrustes analysis on landmark coordinates using the program MorphoJ (Klingenberg 2011). This analysis involves three steps: (1) translation of the landmark configuration of all objects so that they share the same centroid; (2) scaling of the landmark configuration so that they have the same centroid size; and (3) rotation of the landmark configurations to minimize squared Euclidean distances between homologous landmarks (Zelditch et al 2012). In this way, the data matrix of Procrustes coordinates only retains shape information. After superimposition, we averaged landmark configurations from flowers of the same individual. Subsequently, to summarize flower shape variation, we performed a Principal Component Analysis (PCA) on the Procrustes coordinates using the MorphoJ software (Klingenberg 2011). We visualized morphological changes along PCs axes using the transformation grids from the MorphoJ program (Klingenberg 2011).

Using CoordGen 8 software (Sheets 2017), we obtained linear measures from landmark coordinates: the distance between landmark 1 and 8 and the distance between landmark 11 and 18. We then obtained corolla tube length as the average between both distances.

Fitness measures

205 We obtained fruit set and seed number per fruit as female fitness measures. Fruit set (proportion of
 206 pollinated flowers) is an estimate of pollination intensity, while the mean number of seeds per fruit
 207 estimates the quality of mating (Nattero et al 2010a). For each plant, we marked five branches,
 208 recorded the total number of flowers per branch and identified them by painting the persistent calyx
 209 with waterproof paint. After 20–30 days, we counted the number of fruits produced and then
 210 estimated fruit set per plant as the proportion of painted flowers setting fruits. To determine seed
 211 number per fruit, we collected approximately 10 mature fruits per plant shortly before capsule
 212 opening and stored them in paper bags until fruits opened. We weighted a known number of seeds
 213 (ca 200 seeds) and the total seeds collected per plant (all fruits together) with a precision balance
 214 (Sartorius CP224 S, accuracy = 0.1 mg), and estimated the number of seeds per fruit per plant (S_n)
 215 as:

$$216 \quad S_n = S_s \times T_w \times s_w^{-1} \times F_n^{-1}$$

217 where S_s is the number of seeds in the sub-sample, T_w is the weight of all seeds contained in the
 218 fruits, s_w is the weight of the seed sub-sample, and F_n is the number of fruits collected per plant.

219

220 *Analysis of phenotypic variation*

221 To assess differences in floral traits among pollination environments we conducted a nested
 222 analysis of variance (ANOVA) and a Procrustes nested analyses of variance for corolla length and
 223 corolla shape variables, respectively. In both cases, hierarchical levels considered were pollination
 224 environment (hummingbirds, sunbirds and no pollinators), populations within pollination
 225 environments, plants within populations, and flowers within plants (this level was used as residual
 226 variance). In addition, we performed an ANOVA on corolla tube length to detect between-

population differences in this floral trait, using mean values of corolla length from each plant. We conducted these analyses using the R 3.6.3 software (R Core Team 2020).

Analysis of phenotypic selection

We performed separate phenotypic selection analyses for each population and fitness measure (fruit set and number of seeds per fruit). To estimate selection gradients, we used multiple linear regressions following the model proposed by Lande and Arnold (1983). Selection gradients estimate the magnitude and direction of selection on a specific trait or combination of two traits independently from the indirect effect of other traits (Lande and Arnold 1983; Brodie et al 1995). Linear selection gradients (β_i) indicate that extreme phenotypes (minimum or maximum values) attain highest reproductive success. Non-linear selection gradients indicate stabilizing or disruptive selection on one trait (γ_{ii}) or correlational selection on a pair of traits (γ_{ij}). Models included the first two principal components of flower shape and flower tube length as phenotypic measures. We used fruit set and number of seeds per fruit as estimates of reproductive success.

Because regression analyses residuals departed from normality, we applied bootstrap to estimate the significance of selection gradients (Dixon 1993; Benitez-Vieyra et al 2012), following the procedure detailed in Palacio et al (2019). We generated 10,000 bootstrap samples from the original data set. We used selection gradients estimated after each bootstrap to obtain their frequency distributions. We considered a selection gradient was significant if its bias-corrected 95% confidence percentile interval did not include zero. We used the *boot* package of R 3.6.3 software to perform the bootstrapping and estimate the 95, 99 and 99.9% confidence intervals. We provided selection gradient estimates and confidence intervals as supplementary material (S1).

Because the Lande and Arnold's approach represents a linear or quadratic approximation to the relationship between fitness and a given trait or combination of traits (Brodie et al 1995), interpretation of selection gradients may be misleading, especially when a combination of directional and quadratic selection occurs (Mitchell-Olds and Shaw 1987). Hence, we used generalized additive models (GAMs) to depict the relationship between phenotypic traits and fitness, avoiding a priori assumptions about the shape of this relationship. We used binomial error structure for models where fruit set is the response variable. Binomial distribution is the proper choice when response variables is a proportion (Warton and Hui 2011), as it represents the number of times some outcome (in our case the fruits) occurs out of n trials (the flowers, which were either form fruit or not). We used penalized quasi-likelihood estimation, because previous examination detected overdispersion (Zuur et al 2009). Form models where the response variable is the number of seeds per fruit we used negative binomial error structure and maximum likelihood. This distribution is commonly used for count data and, contrarily to Poisson distribution, it can deal with overdispersion (Zuur et al 2009). When we detected significant selection gradients on a trait (either β_i or γ_{ii}), we plotted the relationship between each floral trait and fitness, as estimated from a multivariate GAM, fixing covariates at their mean value. If we detected significant correlational selection gradients (γ_{ij}), we used GAMs to depict the selection surface between those traits and fitness. We obtained bayesian standard errors of prediction according to Wood (2017).

Finally, we used analyses of covariance (ANCOVAs) to examine whether selection patterns (linear, quadratic and correlational) varied significantly among populations, testing the effect of the interaction between the population and phenotypic traits on fitness (Maad and Alexandersson 2004).

Results

Pollinator assemblages

Hummingbird species composition of the pollinator assemblages differed among the native populations. At Piedra Pintada, *Patagona gigas* was the most frequent pollinator followed by *Sappho sparganura* and *Chlorostilbon lucidus*; at Tupiza, *P. gigas* was the only pollinator species, and at Embarcación, *S. sparganura* was the only observed species (Table 2). The bill of these hummingbirds is straight (Fig. 2a-c) with length ranging from 19.37 mm for *Chlorostilbon lucidus* to 44.30 mm for *Patagona gigas* (Table 2). In both South African populations the pollinator assemblages comprised three sunbirds species (*Nectarinia famosa*, *Cinnyris fuscus* and *Cinnyris chalybeus*), but differing in abundance between sites: *N. famosa* was the most frequent species in Buffels River and *C. fuscus* was the most abundant species in Troe troe River (Table 2). The bill of these sunbirds is curved (Fig. 2d-f) with length ranging from 18.23 mm for *C. fuscus* to 35.83 mm for *N. famosa* (Table 2). In the Mallorca populations, we observed only the hummingbird hawkmoth (*Macroglossum stellatarum*) as an occasional legitimate visitor of *N. glauca* flowers.

Phenotypic variation in flower traits

Procrustes nested analyses of variance showed significant differences in flower shape among environments with different pollinators (hummingbirds, sunbirds, and without pollinators; $F_{2,1535} = 191.92$, $P = 0.001$), among populations within pollination environments ($F_{4,1535} = 169.35$, $P = 0.001$) and among individuals within populations ($F_{7,1535} = 3.85$, $P = 0.001$).

The PCA performed with the Procrustes coordinates of the corolla yielded 32 PCs. The first two PCs accounted for 70% of the total variance in shape. The first, PC1 axis (57.53% of variation), was associated with changes in flower width. Low values of PC1 correspond to wide floral tubes

295 and high values correspond to narrow floral tubes (Fig. 3). This PC showed high variation within
296 the native hummingbird-pollination environment. Plants from Embarcación and Tupiza showed
297 high PC1 values (i.e., narrow flowers), while plants from Piedra Pintada reached the lowest PC1
298 values (i.e., the widest flowers). In addition, the latter native population showed the highest intra-
299 population variation in this PC. Plants in the non-native sunbird-pollinated environment showed
300 intermediate PC1 values and plants in the non-native environment without pollinators showed high
301 PC1 values (i.e., narrow flowers). The PC1 variation range across the native environment
302 encompassed variation ranges of both non-native pollination environments.

303 PC2 axis (12.63 % of variation) was associated with changes in both flower curvature and
304 flower width. The PC2 variation ranges differed between the native and non-native pollination
305 environments. Plants in the native hummingbird-pollinated environment showed low PC2 values
306 corresponding to straight and wide corolla tubes, while those in both non-native pollination
307 environments (i.e., sunbird-pollinated and without pollinators) showed high PC2 values which
308 correspond to curved and narrow corolla tubes.

309 We detected significant differences in corolla tube length among different pollination
310 environments ($F_{2,515} = 459.12$, $P < 0.0001$), among populations within pollination environments
311 ($F_{4,515} = 304.94$, $P < 0.0001$) and among individuals within populations ($F_{7,515} = 5.57$, $P < 0.0001$).
312 The variation range of corolla tube length was greater within the native hummingbird-pollination
313 environment than that of both non-native pollination environments and completely encompassed
314 them (Fig. 4). Native populations significantly differ in corolla tube length: plants from
315 Embarcación showed the shortest corolla tubes; plants from Piedra Pintada showed tubes of
316 intermediate length and plants from Tupiza showed the largest corolla tubes. The latter native
317 population also showed the highest variation in this floral trait (Fig. 4). Corolla tube length of plants

from both non-native populations of South Africa (Buffels River and Troe Troe River; sunbird-pollination) did not differ from each other or from that of plants from the native population of Embarcación. Plants from the non-native Mallorcan (i.e. without pollinators) populations of Biniamar and Génova showed values of corolla tube length lower than, and similar to, those of the native population Piedra Pintada, respectively (Fig. 4).

Selection on flower traits

In the selection analyses, we observed significant gradients (linear, non-linear and/or correlational) through both fitness measures in populations of the three studied pollination environments (Fig. 5, Supplementary Material S1).

Through fruit set, we detected negative directional selection on corolla tube length in the native population Embarcación (Fig. 5a): plants with shortest corolla tube length attained higher reproductive success (Fig. 6a). We detected stabilizing selection on PC2 in the native population Piedra Pintada and in the non-native population Biniamar (Fig. 5b and 6b,c), indicating that plants with a flower shape similar to the population mean (straight and wide in Piedra Pintada, and curved and narrow in Biniamar) reached higher reproductive success. We also detected correlational selection between PC1 and PC2 in Biniamar (Fig. 5c). The selection surface (Fig. 7a) showed that the most successful plants had low values of PC1 (i.e., wide corolla tubes) and medium or high values of PC2 (i.e., similar shapes to the population mean and more curved and narrower than the population mean).

Through seed number per fruit, we detected negative directional selection on corolla tube length in Tupiza and positive directional selection on such trait in Biniamar (Fig. 5d and 6d,e). Negative directional selection on PC1 (Fig. 5d) favored plants with flowers wider than the

population mean in Génova (Fig. 6f). We detected negative directional selection on PC2 in Buffels River (Fig. 5d), favoring plants with straighter and broader corolla tubes than the population mean (Fig. 6g). We detected correlational selection through seed number per fruit between PC2 and corolla tube length both in the native population Piedra Pintada and the non-native population Génova (Fig. 5f). The selection surface in Piedra Pintada showed that the most successful plants had slightly high PC2 values (i.e., curved and narrow corolla tubes) and near average or greater corolla tube lengths (Fig. 7b). The selection surface in Génova showed that the most successful plants had low PC2 values (i.e., straight and wide corolla tubes) and short corolla tubes (Fig. 7c).

We examined variation in selection patterns among populations by testing the significance of interaction terms between population and flower traits (Table 3). We found significant interactions between directional selection on corolla tube length and population through both fitness measures (i.e. fruit set and seed number per fruit). For directional selection on PC2, we only found a marginally significant interaction with population through seed number per fruit. Correlational selection involving PC2 and corolla tube length varied among populations in a marginally significant way through seed number per fruit. We did not find any significant interaction between selection on PC1 and population.

Discussion

Variation in flower shape and length

Significant levels of phenotypic differentiation found in both corolla shape and length among native and non-native areas with heterogeneous pollinator assemblages suggest that the pollination context might be an important selection factor. However, trait divergence is also expected due to the effect of random subsampling during colonization of novel areas, i.e. drift, but in this case we

would not expect a match with the pollination environment. Genetic drift occurring during the process of invasion could also be the cause of the loss of variation in both non-native areas (Charlesworth 2003; Dlugosch and Parker 2008; Charlesworth et al 2009; Colautti et al 2010). In addition, the exclusively autonomous reproduction of the Mallorcan populations (Issaly et al 2020) could also lead to a reduction in phenotypic variation.

The high floral variation found among populations within the native range probably suggests the effects of divergent selection mediated by the different pollinator assemblages. In this sense, taking into account different bill lengths of hummingbird species that visit *N. glauca* flowers (Table 2), differences in pollinator assemblages could explain the remarkable variation in corolla length found among populations within the native environment. In fact, native populations with the shortest (Embarcación) and longest (Tupiza) flowers only received visits from a hummingbird with short (*Sappho sparganura*) and long bill (*Patagona gigas*), respectively (see Table 2). In addition, in the Piedra Pintada population, with a pollinator assemblage including three hummingbird species of short and long bills, plants presented flowers intermediate in corolla length. These results agree with previous studies on *N. glauca* in the native range (Nattero et al 2011) and in non-native hummingbird-pollinated populations (Schueller 2007), suggesting that corolla tube length constitutes a pollinator-matching trait in this species. Indirectly, our results suggest that corolla length and shape are not correlated, at least in the native range, because Tupiza showed the longest flowers whereas Piedra Pintada exhibited the widest ones. Only in one non-native population from South Africa (Buffels River) did flower length match the bill length of its most abundant pollinator (i.e., *Nectarinia famosa*, see Table 2). The fact that flower length does not vary between the South African populations is possibly, in part, because pollinator assemblages of both sites involve the same sunbird species, although in different proportions. In addition, their similarity could be due

to a similar origin or to gene flow among populations. In a partial disagreement with our expectations, plants in non-native populations without pollinators did not show the shortest corolla tubes, although they had proportionally narrower flowers than most populations with pollinators. Selection on other floral traits that reduce costs of attraction and reward to pollinators is also expected in the non-native range where local pollinators are absent (Jiménez-Lobato et al 2018). In this sense, nectar volume and sugar concentration differences between Mallorcan, South African and native populations will be subject of future studies in this system.

Unlike flower length and width (PC1), whose variations in non-native populations fell within the range of variation of native populations, corolla curvature (PC2) showed a novel variant in both non-native areas with respect to the studied native populations. As we expected, individuals from the native populations presented flowers with straight corolla tubes, while individuals from all non-native populations presented curved corolla tubes. The fact that hummingbirds at the study populations have straight beaks while the Old World nectar-feeding passerines have relatively curved bills (Paton and Collins 1989; Fig. 2) might explain differences in flower curvature between the native and the South African ranges (Temeles et al 2009). Our result is consistent with that found in another species, where corolla shape varied between populations with different functional pollinator groups (Gómez et al 2008). The curved and relatively narrow corolla tubes found in non-native sites without pollinators could facilitate delayed self-pollination (*sensu* Lloyd and Schoen 1992), that is, when corolla and stamens detach from the gynoecium at the end of anthesis. Consistent with this idea, a previous study on California islands colonized by *N. glauca* (Schueller 2004) found the presence of bent or severely crumpled corollas associated with a higher frequency of spontaneous autogamy. Alternatively, taking into account that corolla curvature improves flower handling during hawkmoth visits (Campos et al 2015), curved and proportionally narrow corollas

in Mallorcan populations could favor pollination by the occasional visitor *Macroglossum stellatarum*. In addition, a study across eight species of *Nicotiana* showed that differences in corolla shape influence flower choice by hawkmoths (Kaczorowski et al 2012). However, instead of being a product of convergent adaptation to their respective pollination environments, a possible explanation for the similarly curved flowers in Mallorca and South Africa is that populations in one of these areas derived from populations in the other, or that both derived from a shared ancestral population outside our study area with curved-flowered plants. Moreover, although part of the variation found in non-native habitats is within the whole range of native populations, there is a chance that those average phenotypes have different origin (e.g. from different native populations). The ongoing genetic work will be very insightful in this regard because it will allow us to assess the genealogy of non-native populations. In addition, genetic analyses will be able to demonstrate whether non-native populations have lower genetic variation (expected by drift and selection, but unexpected if multiple introductions have occurred).

Phenotypic selection

Our results showed significant selection gradients for the three studied floral traits (PC1 and PC2 of floral shape, and corolla tube length) in some native and non-native populations across the three pollination environments. Despite the higher phenotypic variation found within native populations whereupon natural selection can act, we detected a higher number of significant selection gradients in non-native populations. This result is expected if adaptation to the new environment is still incomplete. The fact that current reproductive success of *N. glauca* on both non-native ranges is pollen-limited (Issaly et al 2020) enhances opportunities for selection via female function. We found significant gradients through both measures of female reproductive success, with a higher

number of significant gradients detected through the number of seeds per fruit, suggesting that the effectiveness of pollination affects female fitness more than the proportion of flowers pollinated.

Flower length was the target of selection in five of the seven studied populations, in directional fashion in three of them, and in correlational fashion with PC2 in two populations. In addition, this was the only trait accounting for variation in selection among populations, which was apparent for directional selection through both female fitness measures. Selection favored flowers with corollas shorter than population means in two native populations, although through different fitness measures in each of them. In both cases, population mean corolla length (Figure 3) was higher than bill length of the respective hummingbird pollinators (Table 2), which would explain the negative directional selection pattern found on this trait. Directional selection on flower length was previously reported for native (Nattero et al 2010a) and non-native populations of *N. glauca* pollinated by hummingbirds (Schueller 2007). Contrary to our expectations, we found selection favoring longer corollas in one population and proportionally wider corolla tubes (i.e., lower values of PC1) in the other population of the without pollinators' environment. The occasional visitor *M. stellatarum* did not appear to influence such selection patterns, since the selected corolla length greatly exceeds the length of its proboscis (Faucheux 2013). Instead, longer corollas, together with curved tubes, could facilitate delayed self-pollination in non-native sites without pollinators. Alternatively, selection patterns in this invaded area might be the product of selection unrelated to pollination, as longer and wider flowers structurally allow larger fruits and, therefore, more seeds per fruit, which could improve dispersal success and colonization of the introduced plants (Schueller 2007).

Unlike corolla width (i.e., PC1 of flower shape), which was under selection in only one non-native population of Mallorca, corolla curvature (i.e., PC2) was the target of selection both in

native and non-native populations and in all three pollination environments. Contrary to our expectations, selection favored straight corollas in a non-native sunbird-pollinated population, but stabilizing selection (which would maintain the match between flower shape and pollination environment) was observed in a native and a non-native population without pollinators (i.e., straight and curved corollas, respectively). The evolution of corolla tube curvature also seems to be correlated with the evolution of other traits, as significant correlational selection gradients involved it in combination with corolla length in two populations (one native and one non-native), and with corolla width in one non-native population. The selection on trait combinations only partially agreed with that expected according to the pollination environments. For example, in the Mallorcan population Génova, straight and short corolla tubes were favored, but only the latter pattern agreed with our predictions for the non-native environment without pollinators.

Correspondence between phenotypic differentiation and the spatial variation in selection

Most of the spatial variation found in corolla length and shape supported our expectations based on the pollinator environments. Patterns of phenotypic selection were consistent with floral trait differentiation and with our predictions in some populations. For example, we found negative directional selection on flower length in two native populations (i.e., Tupiza and Embarcación), where the mean value of this flower trait was below the fitness optimum predicted from the corresponding pollinator bill length. In addition, we found stabilizing selection on corolla shape in a native (Piedra Pintada) and a non-native population (Biniamar), where straight and curved floral tubes, respectively, matched the expected patterns according to their pollination environments.

However, selection patterns did not meet all our expectations. In various cases, we did not detect selection on corolla shape or length, while in others the selection patterns found were not

consistent with either floral trait variation or with our predictions. Similarly, another study in *N. glauca* found that selection on corolla length did not explain island-mainland differences in this floral trait in the USA (Schueller 2007). The lack of a correspondence between phenotypic differentiation and selection on floral traits could be because our estimates of natural selection are based on only a single episode of reproduction in a perennial species, not lifetime reproductive success (Endler 1986). In this sense, long-term stability of the selective scenarios is highly unlikely, especially in the invaded range. In addition, selection on floral traits via female function often varies between years (e.g. Conner et al 1996; Caruso et al 2003; Benitez-Vieyra et al 2012), which will impede the response to selection. Alternatively, low heritability could also explain the lack of a response to selection. Although there is significant heritability for corolla length, width and shape in some species (Campbell 1996; Gómez et al 2009), heritability is environment-dependent (Falconer and Mackay 1996) and may be lower in certain populations.

On the other hand, instead of being a product of selection, differences in corolla length and shape among plants of native and non-native populations may be the result of plastic responses to their abiotic environment (Camargo et al 2017). In this sense, given that water stress can reduce flower size (e.g. Carroll et al 2001), primarily because the water cost of large flowers is high (Galen et al 1999), non-native populations in South Africa may have the shortest flowers only because they are growing in the driest environments (see Table 1). Both heritability and phenotypic plasticity of *N. glauca* floral traits are subjects of an ongoing study in native and non-native populations.

Although the reduction in phenotypic variation observed in all non-native populations might reveal the random loss and fixation of alleles in small founding populations (Charlesworth 2003; Dlugosch and Parker 2008; Charlesworth et al 2009), it is unlikely that the apparently

adaptive differences in corolla length and shape among geographical areas are due to stochastic processes such as drift. Instead, the match between floral traits and their pollination environments suggests that current differences in corolla length and shape would seem more likely to reflect a response to varying selection pressures under different pollinator regimes. Selection is likely to vary over time and may have been stronger during a period of pollinator or mate scarcity, such as when *N. glauca* first arrived on non-native areas. It is very difficult to reveal the driving force of long-term adaptation by studying a single selection event.

Acknowledgements

AAC, ANS, SBV and VP acknowledge the National Research Council of Argentina (CONICET) and the Universidad Nacional de Córdoba (UNC) as researchers. We thank the two anonymous reviewers, and associated editor for their valuable suggestions; we also thank MC Baranzelli, G Bertone, R Castro, A Coetzee, MC Díaz-Vélez, MC Maubecin, X Rotllán Puig, C Tur, and C Vignolo for assistance in the field work, and C Vignolo and F Gilles for assistance in the lab. This work was supported by FONCyT (PICT 2015–3089; PICT–2011–0837) to ANS, the Department of Science and Technology of South Africa (438 75946) and the Spanish Ministry of Economy and Competitiveness (CGL2013-44386-P). The authors declare no conflicts of interest.

References

- Benítez-Vieyra S, Medina AM, Cocucci AA (2009) Variable selection patterns on the labellum shape of *Geoblasta pennicillata*, a sexually deceptive orchid. *J Evol Biol* 22:2345-2362
- Benítez-Vieyra S, Glinos E, Medina AM et al (2012) Temporal variation in the selection on floral traits in *Cyclopogon elatus* (Orchidaceae). *Evol Ecol* 26:1451–1468
- Bodbyl Roels SA, Kelly JK (2011) Rapid evolution caused by pollinator loss in *Mimulus guttatus*. *Evolution* 65(9):2541-2552.
- Bookstein FL (1997) *Morphometric tools for landmark data: geometry and biology*. Cambridge University Press, Cambridge.
- Brodie E III, Moore A, Janzen F (1995) Visualizing and quantifying natural selection. *Trends Ecol Evol* 10(8):313–318
- Bromilow C (2001) *Problem Plants of South Africa*. Briza Publications, Pretoria.
- Camargo ID, Nattero J, Careaga SA, Núñez-Farfán J (2017) Flower-level developmental plasticity to nutrient availability in *Datura stramonium*: implications for the mating system. *Ann Bot* 120(4):603-615
- Campbell DR (1996) Evolution of floral traits in a hermaphroditic plant: field measurements of heritabilities and genetic correlations. *Evolution* 50(4):1442-1453
- Campos EO, Bradshaw HD, Daniel TL (2015) Shape matters: corolla curvature improves nectar discovery in the hawkmoth *Manduca sexta*. *Func Ecol* 29:462–468
- Carroll AB, Pallardy SG, Galen C (2001) Drought stress, plant water status, and floral trait expression in fireweed, *Epilobium angustifolium* (Onagraceae). *Am J Bot* 88(3):438-446
- Caruso CM, Peterson SB, Ridley CE (2003) Natural selection on floral traits of *Lobelia* (Lobeliaceae): spatial and temporal variation. *Am J Bot* 90(9):1333-1340

542 Caruso CM, Eisen KE, Martin RA et al (2019) A meta- analysis of the agents of selection on floral
 543 traits. *Evolution* 73(1):4-14
 544 Charlesworth B, Morgan MT, Charlesworth D (1991) Multilocus models of inbreeding depression
 545 with synergistic selection and partial self-fertilization. *Genet Res* 57(2):177-194
 546 Charlesworth D (2003) Effects of inbreeding on the genetic diversity of populations. *Philos T Roy*
 547 *Soc B* 358(1434):1051-1070
 548 Charlesworth D, Willis JH (2009) The genetics of inbreeding depression. *Nat Rev Genet*
 549 10(11):783-796
 550 Colautti RI, Eckert CG, Barrett SC (2010) Evolutionary constraints on adaptive evolution during
 551 range expansion in an invasive plant. . *P Roy Soc B-Biol Sci* 277(1689):1799-1806
 552 Colautti RI, Lau JA (2015) Contemporary evolution during invasion: evidence for differentiation,
 553 natural selection, and local adaptation. *Mol Ecol* 24(9):1999-2017
 554 Conner JK, Rush S, Jennetten P (1996) Measurements of natural selection on floral traits in wild
 555 radish (*Raphanus raphanistrum*). I. Selection through lifetime female fitness. *Evolution*
 556 50(3):1127-1136
 557 Cronk QCB, Fuller JL (1995) Invasive plants: the threat to natural ecosystems worldwide.
 558 Chapman & Hall, London
 559 Dixon PM (1993) The bootstrap and the jackknife: describing the precision of ecological indices.
 560 In: Scheiner SM, Gurevitch J (eds) *Design and analysis of ecological experiments*.
 561 Chapman & Hall, New York, pp 290–318
 562 Dlugosch KM, Parker IM (2008) Founding events in species invasions: genetic variation, adaptive
 563 evolution, and the role of multiple introductions. *Mol Ecol* 17:431–449
 564 Endler JA (1986) *Natural selection in the wild*. Princeton University Press, Princeton

565 Falconer DS, Mackay TFC (1996) Inbreeding and crossbreeding. In: Introduction to quantitative
 566 genetics. Longman Group Ltd, Essex, pp 247–262
 567 Fauchaux MJ (2013) Sensillum types on the proboscis of the Lepidoptera: a review. Ann Soc
 568 Entomol Fr 49(1):73-90
 569 Fenster CB, Ritland K (1994) Evidence for natural selection on mating system in *Mimulus*
 570 (Schrophulariaceae). Int J Plant Sci 155:588–596
 571 Fishman L, Willis JH (2008) Pollen limitation and natural selection on floral characters in the
 572 yellow monkeyflower, *Mimulus guttatus*. New Phytol 177(3):802-810
 573 Galen C (1999) Why do flowers vary? Bioscience 49(8):631–640
 574 Galetto L, Bernardello L (1993) Nectar secretion pattern and removal effects in three species of
 575 Solanaceae. Can J Bot 71:1394–1398
 576 Geerts S, Pauw A (2009) African sunbirds hover to pollinate an invasive hummingbird-pollinated
 577 plant. Oikos 118:573-579
 578 Gómez JM, Zamora R (2000) Spatial variation in the selective scenarios of *Hormathophylla*
 579 *spinosa* (Cruciferae). Am Nat 155:657–668
 580 Gómez JM, Bosch J, Perfectti F et al (2008) Spatial variation in selection on corolla shape in a
 581 generalist plant is promoted by the preference patterns of its local pollinators. P Roy Soc
 582 B-Biol Sci 275:2241–2249
 583 Gómez JM, Abdelaziz M, Muñoz-Pujares J, Perfectti F (2009) Heritability and genetic correlation
 584 of corolla shape and size in *Erysimum mediohispanicum*. Evolution 63:1820–1831
 585 Goodspeed TH (1954) The genus *Nicotiana*. Chronica Botanica Co, Massachusetts
 586 Gunz P, Mitteroecker P (2013) Semilandmarks: a method for quantifying curves and surfaces.
 587 Hystrix 24(1):103-109

588 Herrera CM, Castellanos MC and Medrano M (2006) Geographical context of floral evolution:
 589 towards an improved research programme in floral diversification. In: Harder LD, Barrett
 590 SCH (eds) Ecology and evolution of flowers. Oxford University Press, Oxford, pp 278-294
 591 Hierro JL, Maron JL, Callaway RM (2005) A biogeographical approach to plant invasions: the
 592 importance of studying exotics in their introduced and native range. *J Ecol* 9:5–15
 593 Issaly EA, Sérsic AN, Pauw A et al (2020) Reproductive ecology of the bird-pollinated *Nicotiana*
 594 *glauca* across native and introduced ranges with contrasting pollination environments. *Biol*
 595 *Invasions* 22:485-498
 596 Jiménez-Lobato V, Martínez-Borda E, Núñez-Farfán J et al (2018) Changes in floral biology and
 597 inbreeding depression in native and invaded regions of *Datura stramonium*. *Plant Biol*
 598 20:214-223
 599 Kaczorowski RL, Seliger AR, Gaskett AC et al (2012) Corolla shape vs. size in flower choice by
 600 a nocturnal hawkmoth pollinator. *Funct Ecol* 26(3):577-587
 601 Kingsolver JG, Pfennig DW (2007) Patterns and power of phenotypic selection in nature.
 602 *Bioscience* 57(7):561-572
 603 Klingenberg CP (2011) MorphoJ: an integrated software package for geometric morphometrics.
 604 *Mol Ecol Resour* 11:353–357
 605 Lande R, Arnold SJ (1983) The measurement of selection on correlated characters. *Evolution*
 606 37:1210-1226
 607 Lloyd DG (1987) Allocations to pollen, seeds and pollination mechanisms in self-fertilizing plants.
 608 *Funct Ecol* 1:83-89
 609 Lloyd DG, Schoen DJ (1992) Self- and cross-fertilization in plants. I. Functional dimensions. *Int J*
 610 *Plant Sci* 153(3):358-369

611 Maad J, Alexandersson R (2004) Variable selection in *Platanthera bifolia* (Orchidaceae):
 612 phenotypic selection differed between sex functions in a drought year. *J Evol Biol*
 613 17(3):642–650

614 Mitchell-Olds T, Shaw R (1987) Regression analysis of natural selection: statistical inference and
 615 biological interpretations. *Evolution* 41(6):1149–1161

616 Moeller DA (2006) Geographic structure of pollinator communities, reproductive assurance, and
 617 the evolution of self-pollination. *Ecology* 87:1510-1522

618 Molina-Montenegro MA, Quiroz CL, Torres-Díaz C et al (2011) Functional differences in response
 619 to drought in the invasive *Taraxacum officinale* from native and introduced alpine habitat
 620 ranges. *Plant Ecol Divers* 4:37–44

621 Moragues E, Rita J (2005) Els vegetals introduïts a les Illes Balears. Govern de les Illes Balears,
 622 Palma de Mallorca

623 Murren CJ, Chang CC, Dudash MR (2009) Patterns of selection of two North American native and
 624 nonnative populations of monkeyflower (Phrymaceae). *New Phytol* 183:691-701

625 Nattero J, Cocucci AA (2007) Geographical variation in floral traits of the tree tobacco in relation
 626 to its hummingbird pollinator fauna. *Biol J Linn Soc* 90:657-667

627 Nattero J, Sérsic AN, Cocucci AA (2010a) Patterns of contemporary phenotypic selection and
 628 flower integration in the hummingbird-pollinated *Nicotiana glauca* between populations
 629 with different flower-pollinator combinations. *Oikos* 119:852-863

630 Nattero J, Cocucci AA, Medel R (2010b) Pollinator-mediated selection in a specialized pollination
 631 system: matches and mismatches across populations. *J Evolution Biol* 23:1957–1968

632 Nattero J, Sérsic AN, Cocucci AA (2011) Geographic variation of floral traits in *Nicotiana glauca*:
 633 Relationships with biotic and abiotic factors. *Acta Oecol* 37:503-511

634 Núñez-Farfán J, Schlichting CD (2005) Natural selection in *Potentilla glandulosa* revisited. *Evol*
 635 *Ecol Res* 7(1):105-119
 636 Ollerton J, Watts S, Connerty S et al (2012) Pollination ecology of the invasive tree tobacco
 637 *Nicotiana glauca*: comparisons across native and non-native ranges. *J Pollinat Ecol* 9:85-
 638 95
 639 Palacio FX, Ordano M, Benitez-Vieyra S (2019) Measuring natural selection on multivariate
 640 phenotypic traits: a protocol for verifiable and reproducible analyses of natural selection.
 641 *Isr J Ecol Evol* 1(aop):1-7
 642 Paton DC, Collins BG (1989) Consequences of differences in body mass, wing length and leg
 643 morphology for nectar-feeding birds. *Aust J Ecol* 14:269-289
 644 R Core Team (2020) R: A language and environment for statistical computing. R Foundation for
 645 Statistical Computing, Vienna, Austria. Available from <http://www.R-project.org/> (accessed
 646 April 2020)
 647 Richardson DM, Allsopp N, D'Antonio CM et al (2000) Plant invasions - the role of mutualisms.
 648 *Biol Rev* 75:65-93
 649 Rohlf FJ (2010) tpsDig v2. 16. Free software available. Available from [http://morphometrics.](http://morphometrics.org/morphmet.html)
 650 [org/morphmet. html](http://morphometrics.org/morphmet.html) (accessed May 2017)
 651 Sakai A, Allendorf F, Holt JS et al (2001) The population biology of invasive species. *Ann Rev*
 652 *Ecol Evol Syst* 32:305–332
 653 Schueller SK (2004) Self-pollination in island and mainland populations of the introduced
 654 hummingbird- pollinates. *Am J Bot* 91:672-681
 655 Schueller SK (2007) Island–mainland difference in *Nicotiana glauca* (Solanaceae) corolla length:
 656 a product of pollinator mediated selection? *Evol Ecol* 21:81–98

657 Sheets HD (2017) CoordGen 8. Department of Physics, Canisius College, Buffalo, USA. Available
 658 from <http://www3.canisius.edu/~sheets/morphsoft.html> (accessed May 2017)
 659 Takebayashi N, Wolf DE, Delph LF (2006) Effect of variation in herkogamy on outcrossing within
 660 a population of *Gilia achilleifolia*. *Heredity* 96(2):159-165
 661 Temeles EJ, Koulouris CR, Sander SE et al (2009) Effect of flower shape and size on foraging
 662 performance and trade-offs in a tropical hummingbird. *Ecology* 90:1147–1161
 663 Traveset A, Richardson DM (2014) Mutualistic interactions and biological invasions. *Ann Rev*
 664 *Ecol Evol Syst* 45:89–113
 665 Warton DI, Hui FKC (2011) The arcsine is asinine: the analysis of proportions in ecology. *Ecology*
 666 92(1):3-10
 667 Westerkamp C (1990) Bird-flowers hovering versus perching exploitation. *Bot Acta* 103:366-371
 668 Westley PA (2011) What invasive species reveal about the rate and form of contemporary
 669 phenotypic change in nature. *Am Nat* 177(4):496-509
 670 Wood SN (2017) Generalized additive models: an introduction with R. CRC Press/Taylor &
 671 Francis Group, Boca Raton London, New York
 672 Zelditch ML, Swiderski DL, Sheets HD (2012) Geometric morphometrics for biologists: a primer.
 673 Academic Press, Cambridge
 674 Zuur A, Ieno EN, Walker NJ et al (2009) Mixed Effects Models and Extensions in Ecology with
 675 R. Springer, New York

Figure legends

Figure 1

Location of 18 landmarks used for geometric morphometric analysis of corolla tube shape in *Nicotiana glauca*.

Figure 2

Pollinators of *Nicotiana glauca*. Hummingbirds: (a) *Patagona gigas* on *N. glauca*, (b) *Sappho sparganura* female, (c) *Chlorostilbon lucidus* female. Sunbirds: (d) *Cinnyris chalybeus* male on *Protea nitida* (e) *Nectarinia famosa* male on *P. nitida*, (f) *Cinnyris fuscus* male. Photos: A.N. Sérsic (a), C.C. Maubecin (b), E.A. Issaly (c), A. Pauw (d and e), and D. Shapiro (f).

Figure 3

Biplot based on the Principal Component Analysis (PCA) yielded by geometric morphometric analysis on the corollas of 546 *Nicotiana glauca* plants in three native and four non-native populations. Plants (dots) are colored by population. Ellipses indicate different pollination environments: dotted = native hummingbird-pollination; dashed = non-native sunbird-pollination; solid line = non-native without pollinators. The minimum and maximum scores (i.e. corolla shape) of the first two PCs are shown.

Figure 4

697 Flower length of *Nicotiana glauca* in the seven studied populations. Median values are indicated
698 by the perpendicular line within each box, and dots highlight outliers. Significant differences ($P <$
699 0.05) among populations are indicated by different letters.

700

701 **Figure 5**

702 Selection gradients on *Nicotiana glauca* floral traits through fruit set (a-c) and number of seeds per
703 fruit (d-f). Directional (a, d), quadratic (b, e) and correlational (c, f) selection gradients are
704 indicated. Bars indicate the 95% confidence interval obtained from 10000 bootstrap replicates.
705 Populations are indicated by different colors. Stars indicate significant selection gradients. * $P <$
706 0.05; ** $P < 0.01$; *** $P < 0.001$.

707

708 **Figure 6**

709 Cubic spline regressions between standardized floral traits and reproductive success in native and
710 non-native *Nicotiana glauca* populations where significant directional and quadratic selection
711 gradients were detected. Dotted lines correspond to ± 1 Bayesian Standard Errors.

712

713 **Figure 7**

714 Selection surfaces, estimated through Generalized Additive Models, for pairs of traits significantly
715 affected by correlational selection in three studied populations of *Nicotiana glauca*.

716

Figure 2

[Click here to access/download;Figure;Figura 2.TIF](#)

Figure 4

Figure 5

Figure 6

[Click here to access/download;Figure;Figure 6.eps](#)

Table 1 Geographical coordinates and environmental characteristics: altitude, absolute annual precipitation (AAP), and absolute annual temperature (AAT) of the seven studied populations of *Nicotiana glauca*. Period of fieldwork and number of sampled plants in each population is provided.

Pollination environments and populations	Location	Latitude	Longitude	Altitude (m)	AAP (mm)	AAT (°C)	Period of fieldwork	Number of plants sampled for morphometrics
<i>Native hummingbird-pollination</i>								
Embarcación	Salta, Argentina	23°14'40'' S	64°8'7'' W	310	692	17.2	2013	43
Piedra Pintada	La Rioja, Argentina	29°25'46'' S	67°49'47'' W	1590	338	19.6	2012	75
Tupiza	Potosí, Bolivia	21°40'39'' S	65°34'43'' W	3213	370	11.2	2013	86
<i>Non-native sunbird-pollination</i>								
Buffels River	Northern Cape, South Africa	29°43'13'' S	17°36'53'' E	201	105	18.4	2014	62
Troe Troe River	Western Cape, South Africa	31°36'37'' S	18°43'40'' E	125	145	18.3	2014	80
<i>Non-native without pollinators</i>								
Biniamar	Mallorca, Spain	39°25'48'' N	2°32'60'' E	58	608	16.6	2012	100
Génova	Mallorca, Spain	39°21'1'' N	2°23'25'' E	58	437	18.3	2012	100

AAP and AAT obtained from: Worldwide Bioclimatic Classification System 1996-2015. Altitude obtained from: <https://www.mapsdirections.info/coordenadas-de-googlemaps.html>

Table 2 Pollinator species, percentage of visits and mean bill length (regional mean) of each pollinator species and observation time in three native and two non-native populations of *Nicotiana glauca*.

Pollination environments and populations	Pollinator species	Percentage of visits	Mean bill length ± SD (number of specimens)	Number of 20-min. observation periods
<i>Native hummingbird-pollination</i>				
Embarcación	<i>Sappho sparganura</i>	100	21.16 ± 1.86 (5)	12
Piedra Pintada	<i>Patagona gigas</i>	72.4	44.30 ± 1.02 (4)	12
	<i>Sappho sparganura</i>	45.9	21.53 ± 2.09 (7)	
	<i>Chlorostilbon lucidus</i>	21.8	19.37 ± 0.72 (6)	
Tupiza	<i>Patagona gigas</i>	100	43.89 ± 0.86 (8)	15
<i>Non-native sunbird-pollination</i>				
Buffels river	<i>Nectarinia famosa</i>	66.6	35.83 ± 2.53 (6)	10
	<i>Cinnyris fuscus</i>	25.9	21.26 ± 1.94 (39)	
	<i>Cinnyris chalybeus</i>	7.5	21.22 ± 0.83 (5)	
Troe Troe River	<i>Cinnyris fuscus</i>	55.3	18.23 ± 0.76 (3)	12
	<i>Nectarinia famosa</i>	33.3	30.78 ± 3.17 (582)	
	<i>Cinnyris chalybeus</i>	11.3	20.27 ± 2.99 (1951)	

Table 3 Interaction terms from ANCOVAs between population and slopes (directional, quadratic and correlational gradients) from phenotypic selection models. Significant interactions ($P < 0.05$; in bold) indicate that selection varied across populations.

Fitness measure	Selection gradient	Source of variation	$F_{(1,537)}$	P
Fruit set	linear	Population $\times$ PC1	0.81	0.566
	linear	Population $\times$ PC2	1.13	0.344
	linear	Population $\times$ corolla length	5.24	<0.0001
	quadratic	Population $\times$ PC1	0.39	0.883
	quadratic	Population $\times$ PC2	1.22	0.297
	quadratic	Population $\times$ corolla length	0.89	0.503
	correlational	Population $\times$ PC1:PC2	1.11	0.357
	correlational	Population $\times$ PC1:corolla length	1.05	0.394
	correlational	Population $\times$ PC2:corolla length	0.67	0.677
Seed number per fruit	linear	Population $\times$ PC1	1.20	0.307
	linear	Population $\times$ PC2	2.09	0.053
	linear	Population $\times$ corolla length	2.13	0.049
	quadratic	Population $\times$ PC1	0.27	0.950
	quadratic	Population $\times$ PC2	1.17	0.324
	quadratic	Population $\times$ corolla length	0.20	0.977
	correlational	Population $\times$ PC1:PC2	0.28	0.945
	correlational	Population $\times$ PC1:corolla length	0.64	0.700
	correlational	Population $\times$ PC2:corolla length	2.04	0.059

Click here to access/download
Supplementary Material
S1.xlsx