

A test of the Grant–Stebbins pollinator-shift model of floral evolution

Matthew Moir , Hannah Butler , Craig Peter , Tony Dold and Ethan Newman 

Department of Botany, Rhodes University, Makhanda, 6140, South Africa

Summary

Author for correspondence:
Ethan Newman
Email: e.newman@ru.ac.za

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- Pollinators are thought to play a key role in driving incipient speciation within the angiosperms. However, the mechanisms underlying floral divergence in plants with generalist pollination systems, remains understudied.
- *Brunsvigia gregaria* displays significant geographical variation in floral traits and are visited by diverse pollinator communities. Because pollinators are often shared between populations, we investigated whether specific pollinators are responsible for driving floral divergence between them.
- Three distinct ecotypes were identified, each dominated by three different pollinators: bees, swallowtail butterflies, and long-proboscid flies. Across seven populations, we found a pattern of association between style length and the morphology of pollinators that visit the flowers most frequently and contact the reproductive parts most often. Furthermore, we found significant linear, quadratic and correlational selection on flower number, tepal length and style length within the butterfly- and bee-dominated populations. We also found partial evidence for divergent selection on these traits between experimental sites.
- Our findings suggest that a handful of key pollinators that vary in their importance have the potential to drive population-level divergence in floral traits, which may lead to pollination ecotype formation.

Introduction

‘Since selection is a quantitative process, the characteristics of the flower will be molded by those pollinators that visit it most frequently and effectively in the region where it is evolving. Hence when an evolutionist speaks of a “bee flower” or a “hummingbird flower,” he refers to its relationship with the predominant and most effective vector. He does not mean that the flower is pollinated exclusively by this vector.’

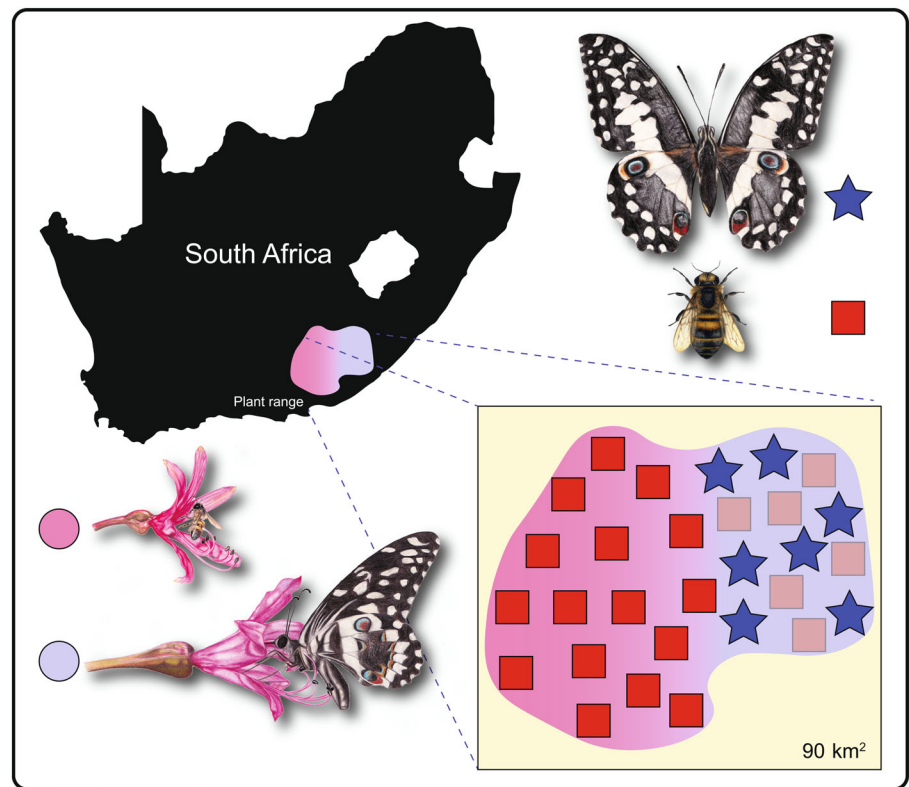
The most effective pollinator principle (Stebbins, 1970).

The incipient stages of pollinator-mediated speciation are well associated with the concept of pollination ecotypes, namely, divergent floral forms of the same plant species adapted to the preferences and morphologies of different functional pollinators across space, time or both (reviewed in Van der Niet *et al.*, 2014a). This concept is well documented and has been the focus of substantial research within the last 25 years (Johnson, 1997; Van der Niet *et al.*, 2014b; Newman *et al.*, 2015; Kay *et al.*, 2019; Castaneda-Zarate *et al.*, 2021; Gross *et al.*, 2023). However, pollination ecotype studies often focus on highly specialised systems wherein distinct floral forms each associate with a specific functional group of pollinators (Johnson, 1997; Anderson *et al.*, 2010; Newman *et al.*, 2012; Peter & Johnson, 2014; Gross *et al.*, 2023). These are ideal model systems for investigating pollinator-mediated speciation, because they depict clear

pollination niches that flowers can adapt to. Oftentimes, however, flowering plant populations interact with a diverse suite of visitors with different preferences and morphologies which may not provide a simple explanation behind how pollinator-mediated adaptive divergence occurs in generalist systems (see Gomez *et al.*, 2014). Indeed, it is widely accepted that pollination generalisation may result in little divergence in floral traits among populations (Waser *et al.*, 1996; Kay & Sargent, 2009). This is based on the assumption that divergent traits of generalist pollinators may exert opposing selective pressures that result in weak net selection on floral traits, both within and between populations (Ohashi *et al.*, 2021). Combined with gene flow from shared generalist pollinators (Gamba & Muchhala, 2023), conflicting selection pressures on floral traits may further limit floral divergence at the population level (Sahli & Conner, 2011).

However, because of spatiotemporal variability in pollinator relative abundances, combined with their behaviours and morphologies that influence floral evolution, there are likely multiple mechanisms associated with pollinator-mediated adaptive divergence in generalist systems (Waser *et al.*, 1996). For example, Aigner (2001) hypothesised that floral traits may adapt to more than one pollinator, leading to the evolution of intermediate phenotypes that occupy distinct adaptive peaks (see fig. 1C in Ohashi *et al.*, 2021). This may occur with or without the fitness

Fig. 1 Grant–Stebbins pollinator-shift model of floral evolution: a conceptual model of floral divergence with reference to the most effective pollinator principle, using a hypothetical example from our study system. *Brunsvigia gregaria* occupies a restricted range in the eastern half of South Africa. Honeybees visit the flowers across the entire range of the plant (pink and light blue regions), however, swallowtail butterflies only occur in one part of the range (light blue region). Where honeybees occur alone (pink region), flowers are smaller and have adapted to the colour preferences and morphology of the bees. Where both bees and butterflies are present (light blue region), butterflies are more efficient pollinators. Namely, they visit the flowers more frequently and transfer larger amounts of outcross pollen, in contrast to bees that transfer poor quality self-pollen, together with outcross pollen. In this region, flowers have adapted to the most efficient butterfly pollinators and have larger flowers matching their larger bodies. Bees, now represented by the pink squares, mismatch the reproductive parts of these flowers and contribute poorly to overall fitness in comparison.



contribution of one pollinator decreasing the fitness contribution from another. In theory, when this occurs across multiple populations that vary in pollinator composition, this may lead to divergent fitness peaks on the adaptive landscape with varying global maxima (Svensson & Calsbeek, 2012), resulting in pollinator-mediated speciation (Schluter, 2000). Related to this idea, it has been recently shown that pollinators of minor effectiveness can still impact net selection on floral traits, where the presence of one pollinator alters selection imposed by the other, potentially affecting spatiotemporal variation in pollinator-mediated divergence (Schiestl *et al.*, 2018; Jaeger *et al.*, 2023).

Historically, in the first conceptual model of pollinator-driven speciation, Grant & Grant (1965) suggested that spatial variation in the relative abundances of different pollinators should lead to divergence in floral traits. Stebbins (1970) is in agreement that the frequency of pollinator visits (i.e. visitation rates) is an important component of pollinator-mediated floral divergence, a measure often used in pollinator-driven speciation studies (Gomez *et al.*, 2014; Parker *et al.*, 2018). However, visitation frequency alone does not provide any information on whether particular visitors transfer more outcross pollen and whether they contribute to overall fitness (reviewed in Ne'eman *et al.*, 2010). Pollinator importance combines both quantitative and qualitative components of pollinator performance (e.g. visitation rates and number of pollen grains deposited) and provides a more accurate picture on the relative contributions of different functional pollinators in the process of pollinator-mediated speciation.

The quality and quantity components of pollinator importance can be directly linked to Stebbins' most effective pollinator principle (Stebbins, 1970), which states that floral traits should evolve through pollinators that visit the flowers most frequently and effectively within an area. This principle is often invoked in generalist pollination systems, where particular pollinators, or a set of functionally similar pollinators contribute substantially more to fitness over others (Wenzell *et al.*, 2023, 2024), and shape floral traits through strong divergent selection.

First suggested by Johnson *et al.* (2006), the Grant–Stebbins pollinator-shift model of floral evolution combines the ideas of Grant & Grant (1965) and Stebbins (1970). It applies the most effective pollinator principle across the distribution range of the species and makes the prediction that if pollinator composition differs in its relative abundances and effectiveness across different parts of the range, it is expected to result in pollination ecotype formation (see conceptual Fig. 1). An additional prediction made by the Grant–Stebbins model is that speciation events may occur through pollinator-mediated isolation (Grant & Grant, 1965; Stebbins, 1970). Surprisingly, however, there is still little evidence of isolating and identifying the most efficient pollinators in pollination ecotype studies (reviewed in Kay & Sargent, 2009), and how, exactly, such pollinators contribute to pollination ecotype formation (but see Schiestl *et al.*, 2018). Contrasting to the notion that generalisation breaks down adaptive floral divergence, pollinators of high importance embedded within generalist systems (*sensu* Waser *et al.*, 1996), may have the potential to drive more among population floral variation than single transitions

between specialist pollinators (Van Valen, 1965), making pollination generalisation largely overlooked as a potential driver of pollinator-mediated speciation (Leménager *et al.*, 2024). This is despite pollination generalisation being widespread amongst the angiosperms (Johnson & Steiner, 2000).

In this study, we use the product of visitation rates and the frequency of pollinator contact with anthers and stigmas to identify the most important pollinators potentially driving floral divergence across *Brunsvigia gregaria* R.A. Dyer (Amaryllidaceae) populations. *Brunsvigia gregaria* exhibits variation in floral morphology across its geographic range in the Eastern Cape of South Africa (Duncan *et al.*, 2017) and is visited by diverse assemblages of pollinators. This forms the basis for the hypothesis that floral variation has likely been driven by local adaptation to differing pollinator assemblages, wherein flower morphology (particularly style length) conforms to the functional body lengths of the most important pollinators across the geographic range of the plant.

This study aims to investigate the Grant–Stebbins model of floral divergence by testing two predictions through field observations and analyses of floral traits. First, through the measurement of functional floral traits, we test the prediction that floral morphology differs significantly between populations. Next, through pollinator observations, we assess the prediction that populations distinguished by divergent floral morphology are also associated with distinct pollinator assemblages. Specifically, we expected to find geographic variation in the importance of different pollinator functional groups, in which style length, a trait likely associated with mechanical fit, is expected to match the size of the most important pollinators (*sensu* Newman *et al.*, 2015). Finally, based on the former predictions, a phenotypic selection analysis focusing on functional floral traits across all phenotype categories, should meet the prediction that *B. gregaria* experiences ongoing divergent selection on floral traits.

Materials and Methods

Study system

The study was conducted over 2 yr (2022 and 2023) during the flowering period of *B. gregaria* and included nine geographically separate populations located between Makhanda and Gqeberha in the Eastern Cape (see Supporting Information Methods S1; Fig. S1). Between March and May, plants produce a single umbel of scentless flowers, which attract insect pollinators (Fig. 2a). The flowers are protandrous, with the anthers dehiscing prior to the stigma becoming receptive. Additionally, anthesis of individual flowers on an inflorescence is staggered. Nectar is housed in a shallow nectar chamber, allowing access to insects with variable body lengths. Pollination occurs when visitors reverse out of the flowers after probing for nectar, contacting the anthers and stigma as they leave (Fig. 2b,c). Alternatively, pollinators such as large butterflies contact the reproductive parts whilst settling on the flowers during foraging (Fig. 2d). Floral visitors with a body length considerably shorter than the length of styles may probe for nectar but miss the reproductive parts of the flower because of a mismatch between pollinator and floral morphology (Fig. 2e). Floral forms identified across all study populations are dependent

on pollinators for their reproductive success and are incapable of selfing, as per a breeding system experiment conducted on all putative floral ecotypes (see Methods S2; Fig. S2).

Floral traits and delineation of ecotypes

To determine whether functional floral traits separate into different floral ecotypes across the distribution range of *B. gregaria*, traits were measured from a total of 482 individuals from all nine study localities (see Methods S1; Fig. S1). From each population, flowers (one from each individual) were cut from the inflorescence at the base of the pedicel, and style length, tepal length and pedicel length were measured using digital callipers. Due to the curling of receptive styles, style length was measured as the straight distance between the stigma and the nectaries during the female phase of anthesis. This is the trait associated with how pollinators match the floral reproductive parts. Next, together with our colour analysis (below), we used these measurements to delineate ecotype populations using a hierarchical cluster analysis (UPGMA) with a Bray–Curtis dissimilarity matrix, which grouped populations using a neighbour-joining tree, independent from the researchers' classification of ecotypes.

Differences in floral morphology between delineated ecotypes were assessed using a PCA, with 95% confidence intervals generated using the *prcomp* function, which was visualised using the *ggplotly* function from the package PLOTLY (Sievert, 2020). Statistical differences between ecotypes occupying the multivariate space were analysed using a permutational multivariate analysis of variance (PERMANOVA) based on Euclidean distances, using the *adonis2* function from the package VEGAN (Oksanen *et al.*, 2013). Pairwise contrasts were computed using the *pairwise.perm.manova* function from the package RVAIDEMEMOIRE (Hervé & Hervé, 2020).

Differences in the functional trait likely to be critical for pollination within this system, style length, were assessed between the three ecotypes generated by the cluster analysis and colour analysis (see the Results section). We ran two models; the first being a generalized linear model (GLM) with a Gaussian distribution, with ecotype representing our fixed factor and style length our response variable. The second model assessed statistical differences among all study populations, using a GLM with population as the fixed effect instead. Both models were computed using the *glm* function from the STATS package and significance of style length variation in association with our fixed effects was assessed using the *Anova (Type 3)* function from the CAR package (Fox & Weisberg, 2018). For the population-level model, a *post hoc* test using the package EMMEANS was used to analyse pairwise contrasts (Lenth & Lenth, 2018).

Spectral reflectance measurements were made at the edge and the centre of the tepal to document the presence of nectar guides from three populations AM ($n = 40$), GE ($n = 50$), and RH ($n = 65$) (see Methods S1 for abbreviated population names) as putative representatives of the short-style, intermediate-style and long-style ecotypes. Measurements were documented using an Ocean Insight FLAME Miniature spectrometer (Ostfildern, Germany) with a PX-2 Pulsed Xenon Light Source and a Premium

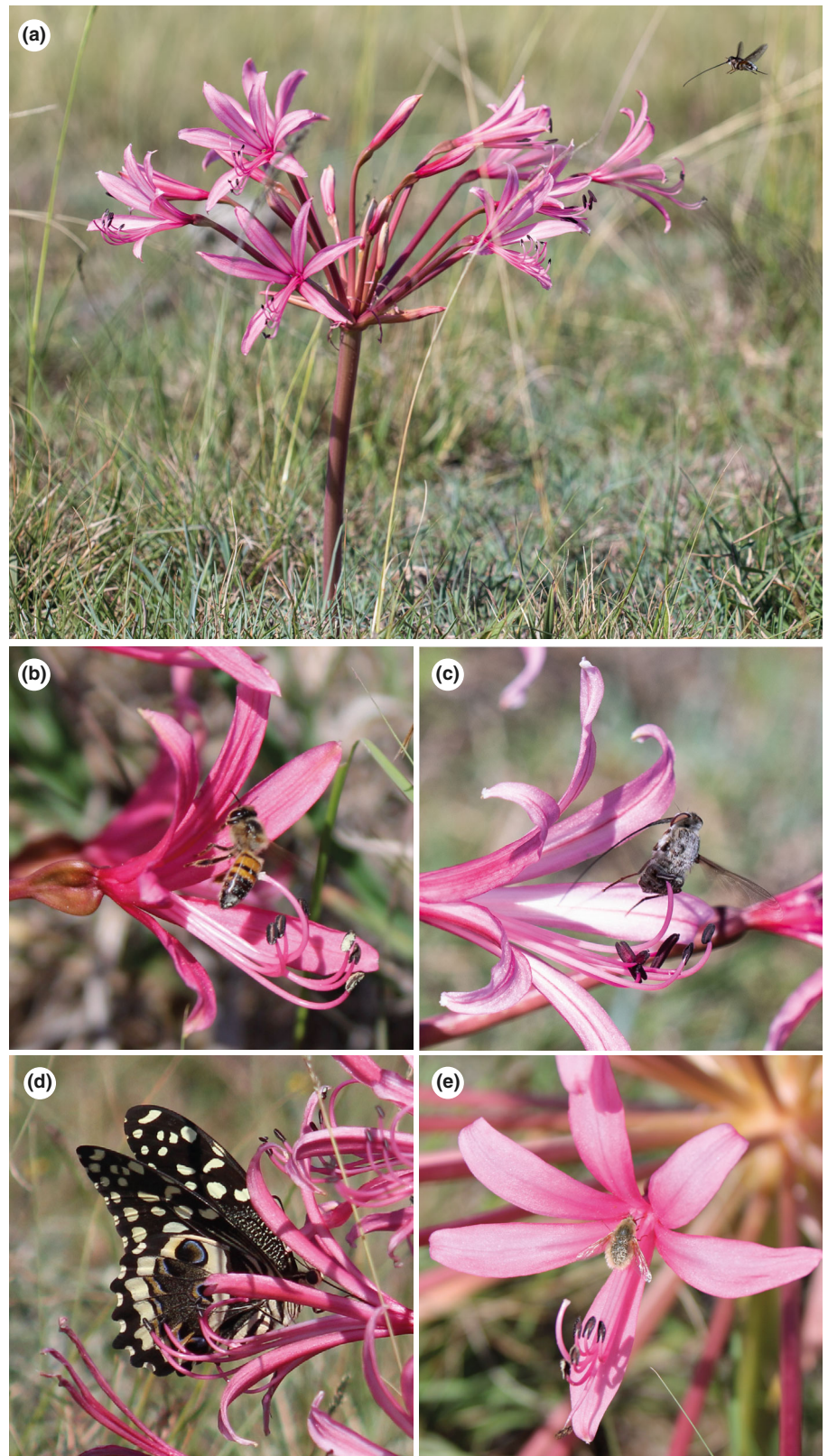


Fig. 2 Images showing *Brunsvigia gregaria* flowers being visited by various insects. (a) *Philoliche aethiopica* approaching the long-style ecotype at Rhini (RH). (b) *Apis mellifera* at the short-style site Paterson (PA). After foraging for nectar, the bee can be seen contacting the style as it reverses out of the flower. (c) *Prosoeca ganglbaueri* at the long-style locality (RH); similarly, the fly can be seen contacting the stigma as it leaves the flower after foraging. Pollen is clearly visible on the thorax and abdomen of the fly. (d) *Papilio demodocus* at the intermediate-style site, Nanaga (NN) rests on the tepals of a flower, allowing the anthers and stigma to contact its thorax and hindwings, as it probes for nectar. (e) *Systoechus vulgaris* is commonly seen foraging for nectar on long-style flowers at RH, yet the pollinators are often too small to contact the reproductive parts.

400 μm fibre optic probe. To visualise the reflectance profiles, aggregated spectral reflectance curves with standard errors were plotted using the *ggplot* function. To further understand how these colours are perceived by different pollinators, we imported

zero corrected spectra into bee, *Apis mellifera* (Chittka *et al.*, 1992), and fly, *Eristalis tenax* (Hannah *et al.*, 2019; Garcia *et al.*, 2022) vision using the *vismodel* and *colspace* functions, using D65 daylight illuminate with a general sample of Eastern Cape foliage

incorporated as background spectra. We did not model our flowers in butterfly perception due to the wide variation in butterfly photoreceptor sensitivities (Arikawa, 2017). All colour analyses were performed using the *PAVO* package (Maia *et al.*, 2019). Euclidian distances relative to the background were calculated for both models; these were visualised as boxplots using *GGPLOT2* (Wickham, 2011), and chromatic contrasts were statistically compared using an ANOVA via the *aov* function and a Tukey *post hoc* test using the *TukeyHSD* function from the *STATS* package.

Geographic variation in pollinator importance

To determine the relative importance of different functional pollinators in the process of pollination ecotype formation, we documented pollinator importance in seven of the nine study sites (see Methods S1; Fig. S1). Across these seven sites, a total of over 50 h were spent observing pollinators between 09:00 and 15:00 h on warm, clear days. The minimum total time spent conducting observations at a particular site was *c.* 3 h and the maximum was *c.* 13 h. Observations were conducted by either one or two observers sitting within one to five meters of the focal plant(s). Between one and five inflorescences were simultaneously observed at a given time. All flower visitors were identified to species level where possible, and up to six individuals of each visiting species from each population were captured. Insects were then measured for their functional body lengths (see Methods S3; Table S1). Apart from long-proboscid flies that vary significantly in proboscis length within < 5 km, functional measurements of Hymenoptera and butterflies of the same species, caught at different sites, were pooled at the species level, because they were unlikely to exhibit significant population-level variation over such small spatial scales (see Newman *et al.*, 2014). These made up the individual components describing our different functional groups.

Pollinator importance Based on their body size and foraging behaviour, we categorised flower visitors into distinct functional groups that are likely to exert similar selective pressures on floral traits (see Methods S3; Table S1). Because functional pollinator groups often consisting of the same pollinator species are present at multiple localities, we calculated locality-specific importance of each pollinator functional group to determine whether pollinator importance varies geographically. The frequency of visits was obtained by calculating the number of flowers visited per inflorescence per hour. For morphological compatibility with styles, we recorded the proportion of times that the pollinators contacted the stigmas or anthers of flowers per foraging bout. These two measurements were independently pooled at each locality, and were used to calculate pollinator importance (PI) for each functional pollinator group (Eqn 1).

$$PI = \text{Visitation rate} \times \text{Frequency of reproductive contact} \quad \text{Eqn 1}$$

A bipartite network, weighted by pollinator importance, was constructed using the *plotweb* function from the package

BIPARTITE (Dormann *et al.*, 2009) to visualise the relative importance of different pollinator functional groups at different localities. A clustering function (Newman's modularity measure: Newman, 2006) was then applied to the network using the package's *computeModules* function to identify modules (i.e. populations that group together based on their most important pollinators). This was repeated for comparison with a network weighted by visitation rate data.

Relationships between flower and pollinator traits Mean functional lengths of pollinators were weighted by pollinator importance at each site. These weighted means were calculated by first multiplying functional lengths by pollinator importance, then dividing the sum of weighted functional lengths by total pollinator importance for each population. This was plotted alongside mean style lengths with their standard errors using *GGPLOT2* (Wickham, 2011). Population means for style length were regressed against weighted mean pollinator functional body length and mean functional body length of the most important pollinator from each locality, separately at each site using *GGPLOT2* (Wickham, 2011). We fitted an ordinary least squares regression to both relationships using the *lm* function in the *STATS* package to assess whether style length is positively associated with pollinator functional body length.

Geographic variation in mechanical fit between widespread occurring pollinators and floral ecotypes If specific functional pollinator groups are responsible for driving divergence within an ecotype, we expect that these same pollinators, when occurring in populations of other ecotypes where they are less effective, should fit those flowers poorly (see Fig. 1). To test this idea, we compared the proportion of contact made to the stigmas and anthers of flowers across multiple populations by the most important pollinators identified by our modules in the network analysis. We made these comparisons between different localities, and across different ecotypes. We did this for medium-sized bees and bee flies for the short-style ecotype, and swallowtails for the intermediate-style ecotype. Because long-proboscid flies only visit long-style plants and occurs within no other ecotype populations, they were not included.

We used GLMs with binomial error distributions and logit link functions. In these models, contact or no contact was assigned as the binary response, and ecotype was assigned as a fixed factor. Models were executed using the *glm* function implemented in the R package *STATS*. Estimated marginal means were obtained from the *emmeans* command in the R package *EMMEANS* (Lenth & Lenth, 2018), which were plotted with asymmetric confidence levels using *GGPLOT2* (Wickham, 2011). This same function was used to calculate pairwise contrasts between ecotypes.

Geographic variation in the form and strength of selection

To assess whether there is ongoing phenotypic selection on functional floral traits across putative ecotypes as generated by the cluster and colour analysis, the largest populations representing different ecotypes, short: Amakhala (AM) and Paterson (PA)

($n = 50$), intermediate: Good Earth (GE) ($n = 105$), and long: Rhini (RH) ($n = 80$), were used to measure phenotypic selection on floral traits. AM and PA were merged as they were in close proximity to one another (< 2 km; Fig. S1). On each inflorescence, the number of pedicels was counted to determine the total number of flowers produced and measurements of tepal and style length were taken for one flower in female phase for each individual. Measured individuals were numbered and tagged. Later, mature infructescences were collected before the dehiscence of fruit. Infructescences were transported to a laboratory setting with the total loss of seeds during transportation amounting to $< 0.5\%$ of the total. The total number of seeds produced by each individual was counted as a proxy for female fitness. Inflorescences exhibiting excessive damage by herbivores were discounted from the experiment. In total, 25 329 seeds were counted, including 9510 from GE, 9409 from RH and 6410 from AM.

Given the discrete nature of the seed set data, we initially used GLMs with a Poisson distribution, considering a log-link function. However, following model diagnostics, we found that all models were overdispersed and so we used GLMs with a negative binomial distribution considering a log-link function instead. Floral traits were standardised to a mean of zero and a variance of one. Linear, quadratic and cross product selection gradients were obtained from these models using custom functions from Morrissey & Goudie (2022), which allows the extraction of selection coefficients from GLMs that specify a log-link function. Significant linear directional selection gradients (β_j) represent a mean increase in fitness towards either end of the trait spectrum, which may be either positive or negative. Significant quadratic selection gradients indicate selection against extreme trait values (γ_{ij}), associated with stabilising selection or selection against intermediate trait values associated with disruptive selection. Another nonlinear selection gradient that measures selection using two traits, correlational selection (γ_{ij}), may be either negative or positive associated with multivariate stabilising or disruptive selection (Walsh & Lynch, 2018). Differences in selection gradients between ecotype populations were assessed using an analysis of covariance (ANCOVA), and a *post hoc* test was conducted using the *glt* function from the package MULTCOMP (Hothorn *et al.*, 2016).

Bivariate fitness surfaces associated with significant correlational selection gradients were generated using thin-plate splines, calculated from the *Tps* command from the *FIELDS* package (Nychka *et al.*, 2015). Fitness surfaces associated with significant directional and quadratic gradients were generated using generalized additive models using a Poisson distribution in association with seed set counts, specifying a log-link function. These models were implemented using the *gam* command in the R package MGCV (Wood, 2017). All statistical analyses were conducted in R (R Core Team, 2022).

Results

Floral traits and delineation of ecotypes

Three ecotypes were identified from the cluster analysis: populations with short reproductive structures, intermediate

reproductive structures and long reproductive structures (Fig. S3). Although AM and PA take a paraphyletic position from one another, we clustered these populations together based on their shared cerise-pink colour, which was distinct from populations with both intermediate and long floral traits, which were similar to each other (Fig. S3).

In support of our findings in the cluster analysis, the PCA of all floral traits revealed that the ecotypes with short and long floral traits form two distinct clusters with no overlap of 95% confidence intervals (Fig. S4). Meanwhile, both forms showed considerable overlap with the ecotype containing intermediate floral traits. The two most important traits in determining these distinctions were style and tepal length, whilst pedicel length accounted for minimal variation. Furthermore, a PERMANOVA confirmed statistical significance in the distinction between the three different ecotypes ($F = 69.3$, $df = 2$, $P = 0.001$; Fig. S4), and all pairwise comparisons were significant ($P < 0.005$). We found statistical differences in style length between ecotypes ($\chi^2 = 1002.8$, $df = 2$, $P < 0.001$), but also between different populations within those ecotypes ($\chi^2 = 1103.5$, $df = 8$, $P < 0.001$). This was associated with variation in the functional lengths of local pollinators (see Fig. 3).

Hence, this analysis confirms the morphological identity of the hypothesised ecotypes. The shortest styles were found at AM (mean \pm SE; 22.7 ± 3.5 mm), followed by PA (27.4 ± 5.4 mm). These two populations were categorised as 'short-style ecotype' according to the cluster analysis and did not differ significantly from one another in their style lengths ($P > 0.05$; Fig. S3). Similarly, style lengths at GE (29.2 ± 5.5 mm), PW (32.3 ± 4 mm), KA (32.9 ± 3.7 mm) and NN (30.1 ± 3.9 mm) did not vary significantly from one another (Fig. 3). Meanwhile, GE, PW and KA all had significantly longer styles than both AM and PA ($P \leq 0.005$; Fig. 3), whilst NN had significantly longer styles than the short-style ecotype from AM, but not PA ($P < 0.001$; Fig. 3). Therefore, GE, PW, KA, and NN were categorised as an 'intermediate-style ecotype' in line with the cluster analysis (Fig. S3). A third, 'long-style ecotype' was identified, comprising three populations that were all found to have significantly longer styles than all short-styled and intermediate-styled populations ($P < 0.0001$; Fig. 3). These were RH (43.5 ± 6.3 mm) and MK (41.7 ± 6.5 mm), with RE having significantly longer styles in comparison to both populations (49.6 ± 8.9 mm; $P \leq 0.01$; Fig. 3).

Aggregated spectral reflectance curves revealed similarity in the colour exhibited by tepals of the intermediate and long-styled ecotypes. By contrast, the short-styled ecotype was distinct in having a lower peak near 400 nm, with the flowers having a cerise-pink colour, in comparison to the other ecotypes with a strong peak in the longer wavelengths (Fig. S5A). The long-styled and intermediate forms both exhibited darker colouration towards the centre of tepals, suggesting the presence of nectar guides (Fig. S5B,C). Little distinction was found between the central and off-centre tepal colour in the short-styled form (Fig. S5D).

Spectral measurements clustered almost entirely within the UV-blue region of the bee colour vision model (Fig. 4a) and Euclidean distances of spectra relative to the background in

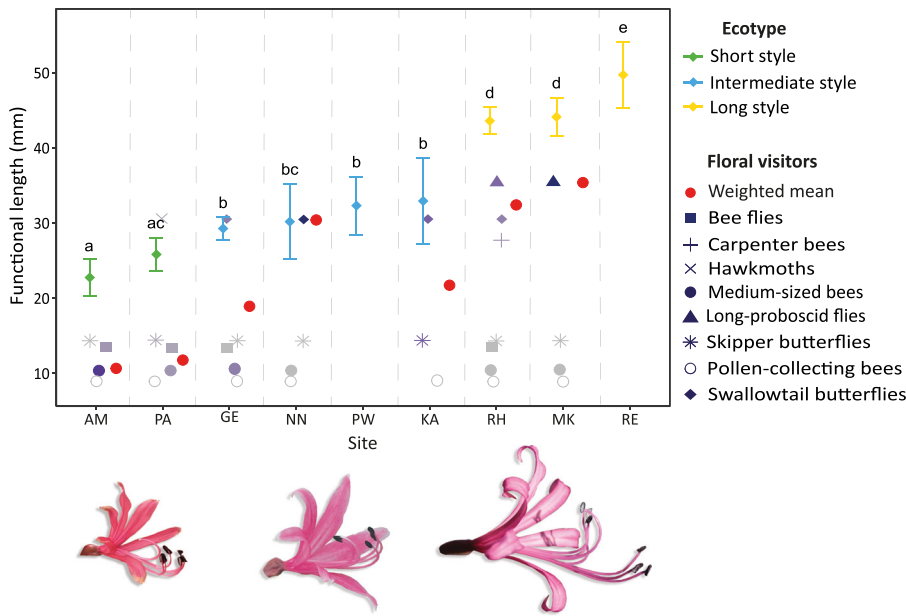


Fig. 3 Style lengths vary across different populations of *Brunsvigia gregaria* in concurrence with geographic variation in the functional body lengths of important pollinators. Style lengths from Amakhala (AM), Paterson (PA), Good Earth (GE), Nanaga (NN), Petworth (PW), Kariega (KA), Rhini (RH), Makhanda (MK) and Riebeeck East (RE) are represented by their means (\pm SE). The mean functional length of each observed floral visitor is represented alongside mean style length, with the intensity of blue representing an increase in pollinator importance. Red circles represent the mean functional length of floral visitors weighted by PI at each site. Significant differences in style length between populations are denoted by different letters.

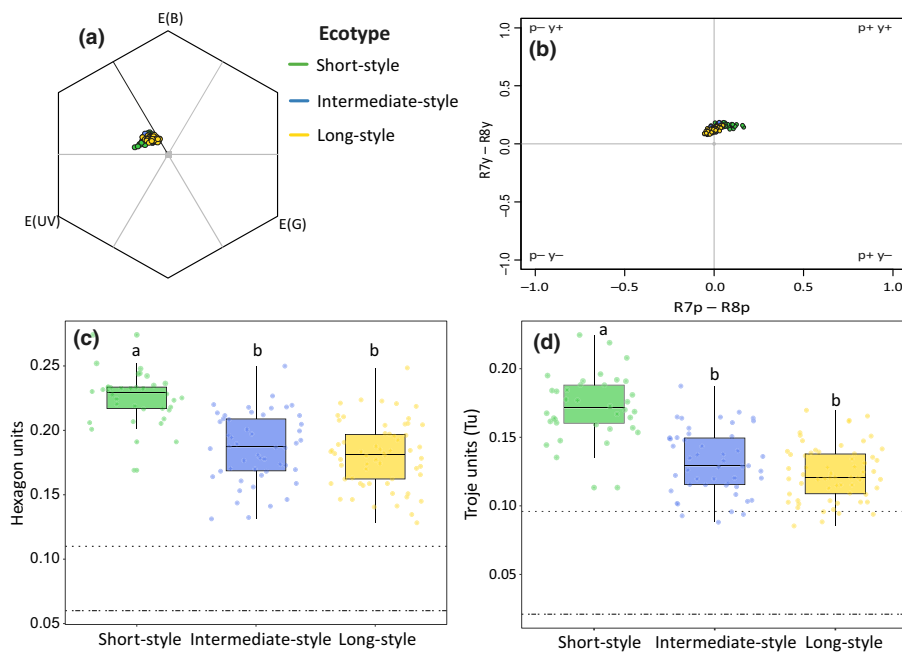


Fig. 4 Floral colouration of *Brunsvigia gregaria* ecotypes as perceived in bee and fly vision. (a) Colour spectra from floral ecotypes plotted in trichromatic bee colour vision (*Apis mellifera*) with UV, blue and green photoreceptors corresponding with E(UV), E(B) and E(G) axes, respectively, and tetrachromatic fly colour vision (*Eristalis tenax*) (b). Euclidean distances from the background colour for all floral ecotypes were calculated for both bee and fly models and are represented graphically with boxplots. All boxplots are well above the minimum perceptibility threshold represented by the dashed lines in both bee and fly vision, with the central line within each box representing the median value. (c) In bee vision, the model suggests that the short-style ecotype is perceived statistically easier than either intermediate or long-style plants from the background. Here, the dashed line represents the minimum threshold of perceptibility at 0.06 hexagon units and the dotted line as easily perceptible at 0.11 hexagon units. (d) Similarly, in fly vision, all floral ecotypes are easily perceived from the background above the dotted line of 0.096 Troje units, which represents the threshold of being easily perceptible. This is also well above the dashed line that represent the threshold of being perceptible at 0.021 Troje units. However, similar to bee perception, short-style flowers were statistically more perceptible than either intermediate or long-style plants from the background colour. Different letters above the boxes denote statistical differences between ecotypes and the dots associated with different ecotypes represent Euclidean distances for each sample measured from the background colour.

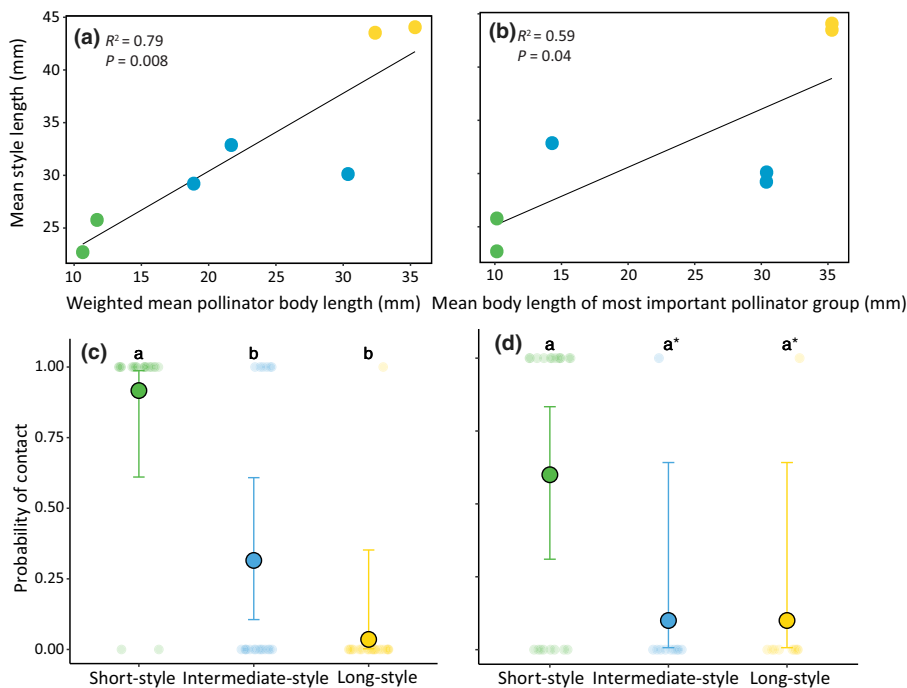


Fig. 6 Plant populations exhibit floral morphology corresponding with their most important pollinators. Meanwhile, as style length varies geographically, ubiquitous pollinators vary in the rates at which they contact the reproductive parts of flowers within different populations. Mean style length is plotted against (a) weighted mean pollinator body length and (b) mean body length of the most important pollinator group at *Brunsvigia gregaria* study sites. (c) Probability of contact made to the anthers and stigmas of flowers by the bodies of honeybees (*Apis mellifera scutellata*) and (d) bee flies (*Systoechus vulgaris*) at their native short-style site, an intermediate-style site and a long-style site. Coloured circles represent the mean probability of contact made by insects to floral reproductive parts, with asymmetric confidence intervals. Different letters represent statistical differences between ecotypes and the dots associated with different ecotypes represent binary data linked with contact or no-contact with anthers and stigmas; asterisks represent probability values near the alpha level of 0.05.

and long-style populations ($P=0.07$; Fig. 6c). Ecotype also had a significant effect of the proportion of contact bee flies made to flowers ($\chi^2 = 11.87$, $df = 2$, $P = 0.003$; Fig. 6d), and pairwise comparisons bordered on significance for the percentage of contact made between short and intermediate-styled populations ($P=0.061$; Fig. 6d) and short and long-styled populations ($P=0.061$; Fig. 6d), but not between intermediate and long-style populations ($P=1.00$; Fig. 6d). Swallowtail butterflies made contact on all flowers visited on both intermediate and long-style ecotypes.

Geographic variation in the form and strength of selection

We found evidence for positive directional selection on flower number at the short-style ecotype localities AM and PA, as well as stabilising selection on style length and positive correlational selection between style length and flower number (Table 1; Fig. 7a). At the intermediate-style site, GE, similarly, we found evidence for positive directional selection on flower number and stabilising selection on tepal length (Table 1; Fig. 7b,c). At the long-style locality, RH, we found no significant selection on any of the documented traits (Table 1). Our ANCOVA suggests significant differences in the selection gradients between the long-style (RH) and the intermediate-style locality (GE) ($t=3.5$, $P=0.001$) and between the short-style localities (AM and PA) and intermediate-style locality (GE) ($t=2.6$, $P=0.02$), but not between the long and short-style localities (RH and AM). AM and PA represent short-style localities, GE our intermediate-style locality, and RH our long-style locality. Linear (β), quadratic (γ_{ii}) and correlational (γ_{ij}) selection gradients are given for tepal length (TL), style length (SL), and the number of flowers per inflorescence (FN). Bold type denotes significant selection gradients and asterisks the significance levels from the associated

generalized linear models (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$). AM, Amakhala; GE, Good Earth; PA, Paterson; RH, Rhini.

Discussion

Our results show partial support for the Grant–Stebbins pollinator shift model. Despite our data not demonstrating pollinator-mediated reproductive isolation in this system, there is support for the initial stages of divergence according to the model. First, we show that the pollinators that are most frequent and make the most contact with the floral reproductive parts closely match floral traits in the populations in which they occur (Figs 3, 6a,b). The most important pollinators also associate with the ecotype groupings as delimited through our multivariate analysis (Figs 5, S3, S4). Furthermore, when these effective pollinators occur outside of the localities where they are most important, they match the reproductive parts of the flowers poorly, suggesting a pattern of local adaptation to the most important pollinators (Fig. 6). Furthermore, we find partial evidence for divergent selection on floral traits when we compare the strength of selection on floral traits between our experimental ecotype localities.

Associations between the traits of the most important pollinators and the flowers they pollinate

Each population was associated with a diverse suite of floral visitors. Interestingly, we found considerable overlap in the presence of certain functional pollinators across different ecotype localities (Fig. 5). Our network analysis considering only visitation rates (Fig. S6) was nested and did not associate well with the different morphological groupings as per our multivariate analysis on floral traits (Figs S3, S4). This set of results reflected those of Gomez

Table 1 Selection gradients (\pm SE) associated with short, intermediate and long-style ecotypes of *Brunsvigia gregaria*. AM and PA represent short-style localities, GE our intermediate-style locality, and RH our long-style locality. Linear (β), quadratic (γ_{ii}) and correlational (γ_{ij}) selection gradients are given for tepal length (TL), style length (SL), and the number of flowers per inflorescence (FN). Bold type denotes significant selection gradients and asterisks the significance levels from the associated generalized linear models (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$). AM, Amakhala; GE, Good Earth; PA, Paterson; RH, Rhini.

Population			Floral traits		
			TL	SL	FN
AM and PA		β	0.062 ± 0.148	-0.071 ± 0.085	$0.502 \pm 0.150^{***}$
	TL	γ	-0.117 ± 0.154		
	SL		0.001 ± 0.105	$-0.436 \pm 0.108^*$	
	FN		-0.118 ± 0.185	$0.206 \pm 0.107^*$	0.219 ± 0.358
GE		β	0.067 ± 0.064	-0.071 ± 0.073	$0.274 \pm 0.07^{***}$
	TL	γ	$-0.232 \pm 0.065^*$		
	SL		0.091 ± 0.067	-0.067 ± 0.097	
	FN		0.071 ± 0.066	-0.093 ± 0.069	-0.057 ± 0.115
RH		β	-0.087 ± 0.101	0.079 ± 0.093	0.116 ± 0.09
	TL	γ	0.040 ± 0.149		
	SL		-0.011 ± 0.137	0.137 ± 0.205	
	FN		-0.132 ± 0.120	0.134 ± 0.141	0.085 ± 0.127

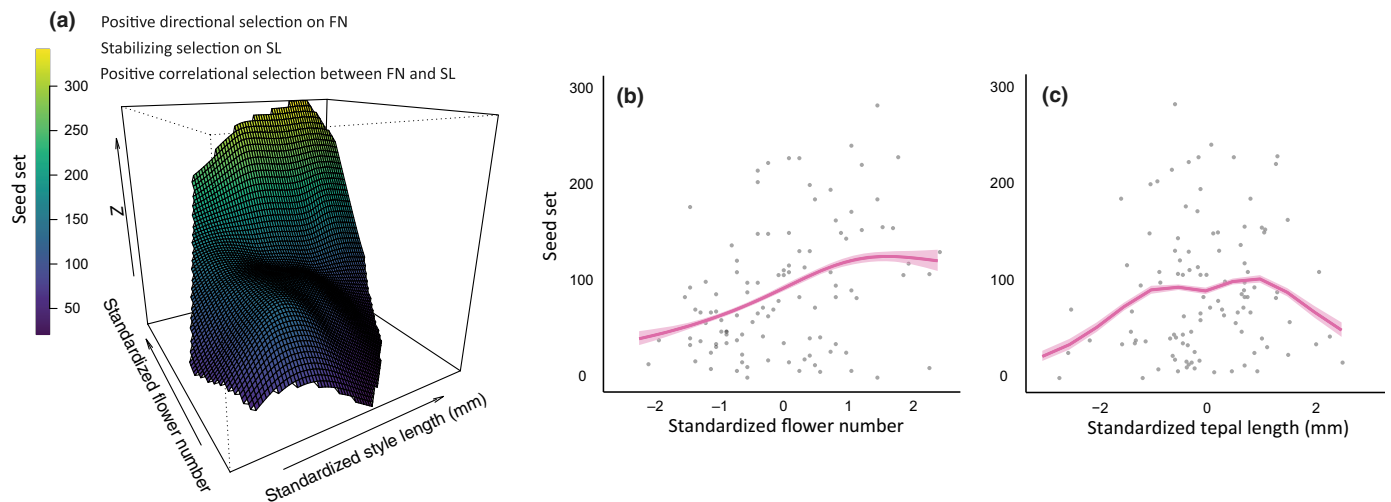


Fig. 7 Fitness functions obtained from generalized additive models associated with significant selection gradients are plotted for (a) the short-style sites, Amakhala and Paterson (AM & PA), and the intermediate-style site, Good Earth (GE) (b, c). See Table 1 for values.

et al. (2014) who used a similar analysis that was weighted by the relative abundances of visitors to *Erysimum mediohispanicum* (Brassicaceae). When we weighted our pollinator network by pollinator importance instead, the connections in the resulting network analysis were greatly reduced. Consequently, we found a clearer association between the most important functional pollinator groups, and *a priori* morphological distinctions generated by our multivariate analysis (Figs S3, S4).

However, even though the connections were greatly reduced in this analysis, we found that the most important pollinators responsible for generating distinct modules were also present in other ecotype localities. For example, medium-sized bees were observed at all sites but were only effective at the short-style ecotype sites where pollinator importance was the highest

(Figs 3, 6c). What is more interesting is that there seems to be a hierarchy with regard to pollinator importance across the different ecotype populations with increasing style length. Specifically, in each ecotype category, we found that the pollinators with the largest bodies had the highest pollinator importance and matched the styles of the local flowers the best (Fig. 3). For example, swallowtail butterflies were effective at both the intermediate and long-style sites, but larger-bodied long-proboscid flies, which were only observed at the long-style sites, matched the long-style flowers more closely (Fig. 3). This trend was documented across all study localities included in our network analysis, suggesting that specialisation is likely to occur within modules, despite pollination generalisation in this system (Thompson, 2005).

This trend in specialisation within modules can potentially be explained by the fact that *B. gregaria* lacks any noticeable morphological filter (e.g. corolla tubes) to block out ineffective pollinators, and style length seems to evolve unidirectionally towards the pollinator that visits it most frequently and makes the most contact with the reproductive parts of the flowers (Fig. 3). With respect to body size, it makes sense that the pollinators with the largest surface areas for plants to place, and receive pollen from, will be the most advantageous with regards to their contribution to fitness. Hence, it is expected that plants should select for the pollinators with the largest body sizes (Aigner, 2005; Newman *et al.*, 2021). However, phenotypic selection on floral traits is strongly influenced by the quality and quantity of the pollen it receives, regardless of pollinator body size. For example, plants are expected to experience higher fecundity with solitary bees that carry high-quality outcross pollen in comparison to honeybees of the same size, which often carry low-quality pollen they receive through visiting several flowers on the same inflorescence (Diller *et al.*, 2022). However, even this reasoning may not be an accurate depiction of how selection acts on floral traits of flowers shared between multiple pollinators. For example, in an experimental evolution study looking at the effects of bumblebees and hoverflies on the evolution of floral traits in *Brassica rapa* over seven generations, Schiestl *et al.* (2018) found that flower morphology did not show evidence of adaptation to pollination by bumblebees which were twice as efficient pollinators as hoverflies. They also did not find selection for intermediate floral traits associated with the preferences and body sizes of both pollinators. Instead, flowers evolved very distinct traits associated with each pollinator species independently.

Nevertheless, our findings support the pattern of local adaptation driven by selection for the mechanical fit between flowers and pollinators (Whittall & Hodges, 2007; Boberg *et al.*, 2014; Newman *et al.*, 2014, 2015; Hollens-Kuhr *et al.*, 2021), and in this study the most important pollinator matches style length the closest. This finding is supported by widespread occurring pollinators that are most important within localities where they fit the flowers the best and make effective contact with the anthers and stigmas, but make poor contact with the reproductive parts of flowers that occur outside of these localities (Fig. 6c,d).

In addition to the mechanical fit between flower and pollinator morphology, significant variation was found in flower colour (Figs 4, S5). The short-style form had very distinct flowers, appearing cerise-pink to human vision and exhibiting less intense spectral reflectance and more uniform tepals in contrast with the intermediate and long-style ecotypes that have light pink tepals with putative nectar guides formed by a darker contrasting midline of each tepal (Fig. S5B–D). Furthermore, this ecotype was found to be significantly more visible to both bee and fly colour vision against a background of foliage. This is consistent with observations that medium-sized bees and bee flies are important pollinators of the short-styled form. Therefore, this colouration may be an adaptation to increase pollinator attraction (Trunschke *et al.*, 2021). Additionally, whilst lepidopteran vision models are not available at the time of

writing, it is possible that the intermediate form exhibits colouration adapted to butterfly preferences. Meanwhile, it is unclear why the long-styled ecotype, ostensibly adapted for long-proboscid fly pollination, appears less distinctive to subjective fly vision. It is, however, possible that the putative nectar guides displayed by long-styled flowers serve an important role in pollinator attraction and are thus adapted to be more distinctive against surrounding tepal colour. Indeed, nectar guides are a common trait in the long-proboscid fly pollination syndrome (Hansen *et al.*, 2012).

Linking effective pollinators with floral divergence

Through hand-pollination experiments, we found all ecotype populations to produce significantly higher seed set in response to our outcrossing treatment (Fig. S2). This suggests that pollinators are important for determining reproductive success and, in turn, driving floral evolution (Kay & Sargent, 2009). It is thus expected that different floral forms experience divergent selection due to geographic variation in insect assemblages and pollinator efficiency (Gómez *et al.*, 2009).

We found selection acting on the studied populations to be relatively weak, likely as a result of conflicting sources of selection, as is expected in a generalised system (Waser *et al.*, 1996; Sahli & Conner, 2011). However, we found positive directional selection for flower number at both the short and intermediate-style localities. This positive relationship between flower number and fitness may be associated with resource availability. However, flower number is suggested to be under pollinator-mediated selection simply because larger displays signal more rewards available to pollinators, and consequently, plants with more flowers receive more visits (Grindeland *et al.*, 2005; Sletvold *et al.*, 2010). Flower size at our intermediate-style site is also likely under positive directional selection for this same reason (Mitchell *et al.*, 2004; Chapurlat *et al.*, 2015). We also found stabilising selection on style length at the short-style site, which is likely a result of adaptation to the morphology of the pollinators in the absence of a restricting corolla tube. This is because reproductive parts that are too long, will not contact the body of the pollinator when they reverse away from the nectar chamber (Fig. 2), and reproductive parts that are too short will likely contact the body of the pollinator where pollen receipt is less optimal. However, understanding the mechanisms underlying pollinator-mediated stabilising selection on style length requires isolating selection exerted by functional pollinator groups of different importance within a site, and studying their individual and additive effects. Further work is required to answer this question. Unfortunately, this is something that we cannot answer with our current analysis.

Despite finding weak selection overall, our selection gradients were found to differ significantly when comparing the intermediate ecotype to the other two forms, thus providing partial support for the hypothesis that our experimental populations are undergoing divergent selection on floral traits. These results are similar to Gómez *et al.* (2009) who found geographic variation in selection regimes within the generalist pollination systems of *E. mediohispanicum*. Their findings suggest that selection is

context-dependent on the interactions between pollinators and herbivores within a site.

Conclusions

Three putative ecotypes, a short-style, intermediate and long-style form, were identified based on significant style length differences and were associated with the most important pollinators. Suites of morphological traits were particularly distinct between the short-style and long-style ecotypes, which both display similarities with the intermediate form. However, because the intermediate-style form has a distinct important pollinator, it likely represents a separate adaptive peak, from short and intermediate-style forms in the adaptive landscape. Evolutionary change of this nature is expected where selection on floral traits are driven by subtle changes in pollinator community structure rather than discrete pollinator shifts. Future research should try to isolate pollinator-mediated selection exerted by different functional pollinators, and across several localities, to better understand how divergent selection is possible when multiple pollinators are involved (Schiestl *et al.*, 2018).

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Competing interests

None declared.

Author contributions

EN conceptualised the study and supervised MM. EN, MM and HB did the fieldwork. MM wrote the first draft. MM and EN wrote the paper. HB, TD and CP provided input on the manuscript and guidance on the study system.

ORCID

Hannah Butler  <https://orcid.org/0000-0002-5865-2306>
 Matthew Moir  <https://orcid.org/0000-0002-0906-5375>
 Ethan Newman  <https://orcid.org/0000-0002-9678-4895>
 Craig Peter  <https://orcid.org/0000-0002-9741-6533>

Data availability

The data is available via the following link through DRYAD doi: [10.5061/dryad.2280gb637](https://doi.org/10.5061/dryad.2280gb637).

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Supporting Information

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

Fig. S1 Map of *Brunsvigia gregaria* localities and study sites representing distinct ecotypes.

Fig. S2 Proportion seed set recorded for each ecotype, resulting from hand-pollination treatments conducted during a breeding system experiment.

Fig. S3 Hierarchical cluster analysis based on floral trait means recorded at each study site.

Fig. S4 Principal components analysis showing clustering of ecotypes in the multivariate space based on floral trait measurements.

Fig. S5 Aggregated spectral reflectance curves showing overall spectral profiles and within-flower spectral contrasts (i.e. putative nectar guides) for the different ecotypes.

Fig. S6 Bipartite network and modules computed based on pollinator interactions weighted by visitation rate.

Methods S1 Description of study localities.

Methods S2 Description of hand-pollination treatments, data analysis and results from a breeding system experiment.

Methods S3 Description of pollinator functional length measurements and how pollinator species were assigned functional groupings.

Table S1 Total visits, visitation rates, contact rates, pollinator importance and mean functional lengths for each pollinator functional group at each study site.

Table S2 Visitation rates at each study site for each pollinator species recorded during observations.

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