

Character displacement drives floral variation in *Pelargonium* (Geraniaceae) communities

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Interactions between plant community members are an underexplored driver of angiosperm floral variation. We investigate character displacement as a potential contributor to floral variation in *Pelargonium* communities. *Pelargonium*s all place pollen on the ventral sides of their pollinators, potentially leading to interspecific pollen transfer (IPT) in sympatry. We show that the positions of pollen placement and receipt are determined by anther and style exertion lengths. Using field experiments, we demonstrate that heterospecific species experience higher IPT if they have similar style lengths than when they have greater style length differences. Using crosses, we show that IPT has negative consequences on seed set. In combination, these results suggest that character displacement in style length is likely to reduce IPT and increase female fitness in sympatry. Patterns of style length variation across 29 different *Pelargonium* communities suggest that character displacement has occurred in multiple communities. Furthermore, analyses using a wide-ranging species pair show that style lengths are more different between sympatric populations than they are between allopatric populations. In addition to pollinators as agents of floral divergence, this study suggests that variation in *Pelargonium* community structure has driven style length variation through character displacement.

KEY WORDS: character displacement, community ecotypes, interspecific pollen transfer, long-proboscid fly, pollen placement, reproductive interference.

It is the most closely allied forms—varieties of the same species and species of the same genus or related genera—which from having nearly the same structure, constitution, and habits, generally come into the severest competition with each other (Darwin 1859).

Antagonistic interactions have long been thought of as important drivers of organismal diversification (Darwin 1859; Brown and Wilson 1956; Levin 1970; Grant 1972; Schluter 2000a; Pfennig and Pfennig 2012). In particular, ecological character displacement (ECD) can occur when phenotypic divergence evolves to reduce competition for resources (Slatkin 1980; Dayan et al. 1989; Grant 1994; Adams and Rohlf 2000; Losos 2000; Marchinko et al. 2004; Calsbeek and Cox 2010; Kooyers et al. 2017; Eisen and Geber 2018). Phenotypic divergence can also evolve to reduce reproductive interference and is then termed reproductive character displacement (RCD; Levin 1985; Kawano

2002; Kirschel et al. 2009; Hopkins 2013; Stanger-Hall and Loyd 2015). Regardless of the process, character displacement is thought to generate geographically variable phenotypes, which may facilitate speciation events through the evolution of reproductive isolation (Schluter 2000a,b; Pfennig and Pfennig 2012).

Antagonistic interactions may be at the heart of some of the most impressive species radiations (Losos et al. 1998; Losos 2000), and to understand these, it is important to study how antagonism gives rise to both phenotypic divergence and reproductive isolation. For example, using a representative species from the largest tetrapod amniote genus (Uetz et al. 2019), Calsbeek and Cox (2010) created new island populations of *Anole* lizards and demonstrated that competition rather than predation was the primary agent of selection on morphological traits associated with niche divergence in *Anole* island radiations (Losos et al. 1998). The link between antagonism, morphological divergence, and

reproductive isolation was demonstrated in closely related *Pogonilus* tinkerbirds, which diverged in body size, bill morphology, and mating calls when they co-occurred in sympatry (Kirschel et al. 2009). Divergence in these traits alleviated reproductive interference between co-occurring species but simultaneously generated pre-mating barriers in sympatry.

Often putative cases of antagonistic divergence are first identified by recognizing geographic patterns associated with character displacement. The hallmark pattern associated with character displacement is that the traits of co-occurring species should be divergent in sympatry; however, because those species do not interact in allopatric populations, those same traits can be very similar in allopatry (see fig. 2 of Grant 1972). In addition to character displacement, this pattern (trait overdispersion) can also reflect nonadaptive species sorting where community assembly is dictated by the compatibility of pre-existing phenotypes (Gause et al. 1934; Connell 1961). Consequently, to distinguish between character displacement and ecological sorting, one needs to look at more than just patterns of trait overdispersion. Notably, one needs to show that antagonistic interactions are the likely cause of the overdispersion patterns (see Adams and Rholf 2000). As originally noted by Darwin (see quote above), the more similar the traits of interacting species, the stronger the fitness consequences of competition or reproductive interference in sympatry (Pfennig and Pfennig 2010). Consequently, a key element of demonstrating character displacement (in addition to patterns of overdispersion) is that trait differences should alleviate competition or reproductive interference (reviewed in Germain et al. 2018). Despite the large number of apparent character displacement studies, the link between overdispersion and competition or reproductive interference is seldom well demonstrated (Stuart and Losos 2013). A review by Stuart and Losos (2013) found that while 70% of character displacement studies ($n = 144$) demonstrated overdispersion patterns, a mere (17%) made the link between these patterns and a potential mechanism (see fig. 2 of Stuart and Losos 2013). There is a particular dearth in character displacement studies on plants, with only 11 plant studies displaying some evidence for character displacement mediated through pollinators (see table 1 of Eisen and Geber 2018).

In this manuscript, we study character displacement in *Pelargonium* (Geraniaceae) communities. Eight *Pelargonium* species frequently co-occur across our study sites and all are pollinated by functionally similar long proboscis flies, *Prosoeca longipennis* and *P. ganglebauri* (Manning and Goldblatt 1995; Newman et al. 2014). Plants pollinated by these flies all share similar attractive traits (Newman et al. 2014), and flies do not demonstrate floral constancy in *Pelargonium* communities (Ethan Newman unpublished data). *Pelargonium*s visited by these flies have a long tube (hypanthium) with nectar at the base. Although *Pelargonium* tube length and fly proboscis length are usually

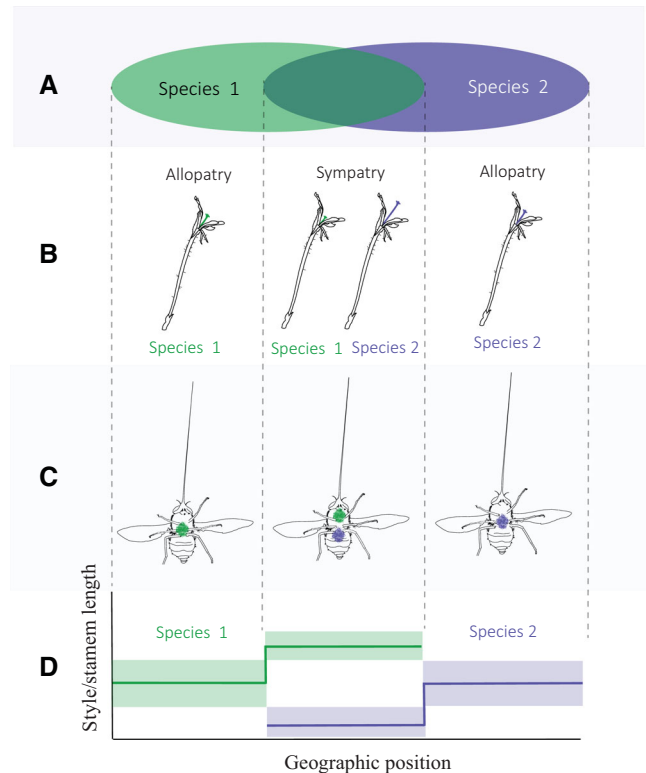


Figure 1. Hypothetical illustration demonstrating style and stamen length divergence driven by IPT. (A) The distribution ranges of species 1 (green) and species 2 (blue) are largely allopatric, but overlap in the middle. (B) When species 1 and species 2 occur in allopatry (left and right), they can have similar style and stamen lengths. However, when they occur in sympatry, they evolve differences in style and stamen lengths to reduce IPT. (C) Evolved differences in style and stamen lengths result in pollen placement and receipt on different parts of a pollinator's body, hence reducing IPT. (D) The expected outcome of character displacement on style/stamen length in sympatry versus allopatry.

closely matched, *Pelargonium* tube lengths are almost always slightly longer than the proboscis of their long proboscis fly pollinators (Newman et al. 2014), a trend that appears to hold for most long-tubed plants and their pollinators (Anderson et al. 2010). Consequently, visiting flies insert their entire proboscis into the hypanthium when foraging and the degree of stamen/style exertion from the mouth of their floral tubes likely determines where contact is made with the ventral surface of the pollinator's body (Fig. 1).

Here, we examine the hypothesis that similarity in *Pelargonium* floral reproductive parts causes reproductive interference, which leads to the evolution of character displacement. To address this hypothesis, we test the following assumptions and predictions: (1) The positions of pollen placement and receipt are determined by anther and style exertion lengths. (2) Style length similarity among interacting heterospecifics leads to more

interspecific pollen transfer (IPT) than if style lengths were different. (3) IPT has a negative effect on seed production. (4) Style lengths of species in sympatric populations are divergent in comparison to those in allopatric populations, which may frequently overlap. The pattern of character displacement should occur repeatedly for several communities and species, making it unlikely to be chance occurrence.

Methods

DOES STYLE LENGTH SIMILARITY LEAD TO REPRODUCTIVE INTERFERENCE?

We chose two *Pelargonium* communities to examine whether style length similarity is associated with increased IPT. The two communities chosen were Bosfontein (−34.036021°, 21.456610°) and Suurbraak (−34.017767°, 20.597721°), which are separated by a straight-line distance of 90.55 km. Both localities are situated on the foothills of the Langeberg Mountains in the Southern part of the speciose Cape Floristic Region (CFR) of South Africa. These localities were chosen because they share the same pollinator species, similar floral guild community composition, and similar habitats (see Newman et al. 2014). In addition, visits by pollinators were consistent within these communities, and the *Pelargonium* species within those communities were known to overlap slightly in the positions of their reproductive structures and have pollen that was distinguishable under a light microscope. One important difference between these two sites is that one site (Bosfontein) has two co-occurring *Pelargonium*s, whereas the other site (Suurbraak) has an additional species (three species).

Stamen and style exertion length

Pelargonium species in this study are protandrous, with separate male and female phases. The life of a flower is usually between 3 and 5 days starting with pollen release (male phase) and ending with stigmatic receptivity (female phase) (Ethan Newman unpublished data). To quantify overlap in anther exertion, we measured the exertion distance for the least and most exerted anthers in a single flower per plant. Measurements were made from the flower gullet where the hypanthium sepals flare, to the start of the closest anther and to the tip of the most distant anther. Only mature, dehisced anthers were measured. We termed these measurements as “minimum” and “maximum” anther exertion lengths. We measured anther exertion lengths of 41 *P. pinnatum* and 57 *P. carneum* individuals at the Bosfontein site, and 39 *P. pinnatum*, 17 *P. dipetalum*, and 12 *P. carneum* individuals at Suurbraak. These anther exertion lengths likely determine where pollen is placed on the body of a pollinator (see Fig. 2).

If style exertion length determines where pollen is received from a pollinator’s body, IPT is expected to increase with increas-

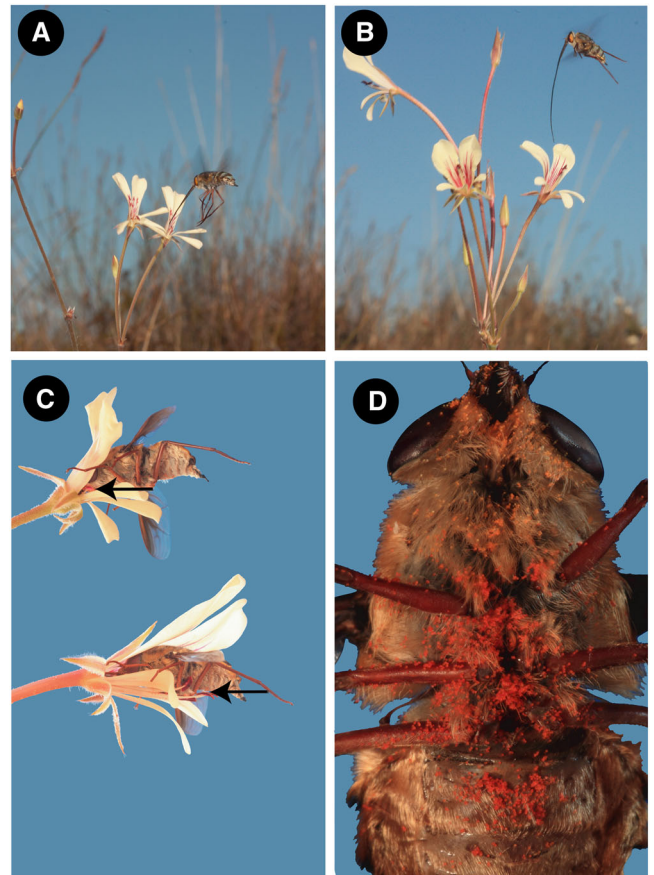


Figure 2. *Pelargonium* species share similar “attractive” morphological traits and differ mainly in the exertion of their stamens and styles, which aids in differential pollen placement and receipt (A–D). At Bosfontein, *P. longipennis* visits two *Pelargonium* species, *P. pinnatum* with shortly exserted reproductive parts (A) and *P. carneum* with more pronounced exertion of reproductive parts (B). Differences in stamen exertions (arrows point to anthers) result in different pollen placement sites on pollinators. *Pelargonium pinnatum* (top) places pollen underneath the insects’ head, whereas *P. carneum* (bottom) places pollen mainly on the middle of the fly’s body (ventral side) (C). *Pelargonium pinnatum* pollen is identified by its light-yellow color underneath the head, eyes, and first section of the mesothorax. In contrast, *P. carneum* pollen grains are a deeper orange color, larger, and more rounded than the *P. pinnatum* pollen grains. Relatively few are found on the head region of the pollinator and are mainly on the middle of the fly’s body (D). Photographs: Ethan Newman (A and B), Bruce Anderson (C and D).

ing overlap in style length. Style exertion length was measured from the flower gullet to the tip of the most distant receptive stigma lobe. Styles were measured from a single flower per plant. At Bosfontein, we measured styles from 65 *P. pinnatum* and 94 *P. carneum* individuals. At Suurbraak, we measured the styles of 53 *P. pinnatum*, 34 *P. dipetalum*, and 23 *P. carneum* individuals.

Comparisons of stamens and styles within localities: To assess statistical differences in anther and style exertion lengths within localities, Mann-Whitney *U*-tests were used at Bosfontein. One-way ANOVA's with post hoc Tukey's honestly significant difference tests (HSD) were used to compare style and anther exertion lengths at Suurbraak, except for one instance where a nonparametric Kruskal-Wallis test was used.

Comparisons of stamens and styles between localities: To compare anther and style exertion lengths between the Bosfontein and Suurbraak localities for both *P. pinnatum* and *P. carneum*, we used Student's *t*-tests. We expected that the style and anther exertions of *P. pinnatum* and/or *P. carneum* would shift (relative to Bosfontein) with the presence of an additional species (*P. dipetalum*) at Suurbraak.

Pollen placement

To determine where different *Pelargoniums* place their pollen, seven flies were captured from Bosfontein and four were captured from Suurbraak. Sample sizes were low because although visitation was consistent, populations of pollinators are small at both localities. Furthermore, flies were only killed if we were able to transfer them into a killing jar without touching them with the side of the net. This was done by covering foraging flies with the net and holding the narrow part of the net to form a "tent." The killing jar was then slipped underneath the net and the fly captured inside. Killing jars contained a strong concentration of Potassium cyanide fumes, which killed the flies almost instantly.

Immediately after being killed, the flies were pinned with their ventral surfaces facing upward. Later the same day, we divided the bodies of individual flies into 2mm intervals using a microscope graticule, starting from the base of the proboscis and ending at the tip of the abdomen. At each interval, the total number of pollen grains was counted under a dissecting microscope and each pollen grain was assigned to different *Pelargonium* species using color, size, and shape as diagnostic features. Prior to the experiment, using 25 flowers from different individuals per species, we ascertained that pollen could be distinguished based on the following characters: at Bosfontein, *P. pinnatum* always has light yellow pollen, which is smaller and more ovate than the deep orange to brick red pollen of *P. carneum*. *Pelargonium pinnatum* pollen at Suurbraak is identical in appearance to *P. pinnatum* at Bosfontein but differs from *P. dipetalum* pollen at Suurbraak, which is always brick red. Although color could be used to distinguish *P. carneum* pollen (orange-pale yellow) from *P. dipetalum* pollen (brick red) at Suurbraak, we could not use color to distinguish *P. pinnatum* from *P. carneum* pollen. Instead, we used shape and size characters, which differed in the same way as described for the Bosfontein site. On each of the captured flies, we recorded the species identity of each pollen grain and its distance

(to the nearest 2mm) from the base of the fly's proboscis (pollen placement site). We used this information to make comparisons of pollen placement site within and between the two localities.

Comparisons of pollen placement sites within localities: To determine whether *Pelargonium* species occupy different pollen placement sites on flies, we used generalized estimating equations specifying gamma error distributions and logarithmic link functions for right-skewed pollen placement site data. An exchangeable correlation structure accounted for flies as repeated subjects. Pollen placement sites were treated as dependent variables and species as a fixed factor. Post hoc pairwise comparisons were performed with a Sequential-Šidák correction at Suurbraak.

Comparisons of pollen placement sites between localities: Here, we tested whether the presence of *P. dipetalum* at the Suurbraak site is associated with a shift in the peak area of pollen placement, for either one of the species (*P. pinnatum* or *P. carneum*) that occurs at both the Bosfontein and Suurbraak sites. For between site comparisons, we used the same statistical methods as explained above, with locality, with locality as a fixed factor. We used SPSS 25 (IBM Corp) for all the above analyses.

Style length overlap and IPT: In both communities, we conducted experiments to determine whether the proportion of IPT increased as style length similarity among species increased. We expected that the more dissimilar style lengths are among species, the more conspecific or "pure" pollen would be transferred to stigmas. Under the next two subheadings, we document the methods of two pollen purity-style length correlation experiments: one using existing, local style length variation and another where local style length variation was increased using flowers translocated from other populations.

Pollen purity-style length correlations using local variation: At Bosfontein and Suurbraak, all experimental inflorescences were cut from the focal populations after their anthers had dehisced, but before stigmas were receptive. Cut inflorescences were kept in a cool room until the stigmas became receptive (strongly lobed and coarsely pustulate). At this stage, the anthers have already dehisced and have dropped from the flower. We removed all other flowers and developing fruits from the inflorescence so that only a single flower per inflorescence was offered to foraging pollinators at each locality. Before we set out inflorescences in the field, we measured and recorded style lengths on tags attached to the pedicel of each flower. This enabled us to associate style length of each flower with pollen deposition, which would be collected later.

On the 6th and 7th of March 2013 at Bosfontein and 27 and 28 March 2014 at Suurbraak, experimental plants were placed in water-filled test tubes and mounted on skewer sticks of equal length. These were placed in the field early in the morning before pollinators were active. Numbers of experimental plants roughly

reflected the ratios of co-occurring species in the natural communities where flies visited both species indiscriminately (Ethan Newman unpublished data). At all localities, *Pelargonium* species can be completely intermixed (within centimeters of one another) and so experimental plants were spaced approximately 30 cm apart where pollinators could have access to experimental plants within an area of approximately 10 m². Although pollinators are scarce, they reliably visit every day and a single pollinator is likely to visit a large proportion of the flowers in the population.

At Bosfontein, 66 individuals of *P. pinnatum* and 33 individuals of *P. carneum* were placed in the field. At Suurbraak, 39 native individuals of *P. pinnatum*, 61 individuals of *P. dipetalum* (40 native and 21 introduced), and 51 individuals of *P. carneum* (32 native and 19 introduced) were used.

Pollen purity-style length correlations using introduced variation: Because selection may have eliminated the variability required to detect relationships between style length and pollen purity, we introduced plants to increase the degree of stylar overlap between *P. carneum* and *P. dipetalum*. This was done at a single community (Suurbraak). *Pelargonium carneum* plants were introduced from Bosfontein with style exertions shorter (13.77 ± 0.24 [mean \pm SE], $n = 65$) than at Suurbraak (15.45 ± 0.24 [mean \pm SE], $n = 23$). *Pelargonium dipetalum* were introduced from locality 19 (-34.016607° , 20.462468°) near the town of Swellendam, which is 13km from Suurbraak. Here, plants had longer styles (13.98 ± 0.2 [mean \pm SE], $n = 28$) than *P. dipetalum* plants at Suurbraak (11.00 ± 0.27 [mean \pm SE], $n = 44$). Similar to the experimental procedures using local stylar variation, introduced individuals had longer hypanthium lengths than the fly proboscis lengths in the experimental populations (see Newman et al. 2014). Consequently, flies insert their entire proboscis into the introduced flowers to obtain nectar and visit the flowers in the same way as they visit the native flowers.

All inflorescences from both experiments were collected at 16:00 and transported back to the field station where we immediately started identifying pollen on stigmas. For each stigma, we recorded the number of conspecific and heterospecific *Pelargonium* pollen grains. Flowers that were not visited and still fresh were used again the following day (petals quickly fall off flowers after they receive pollen from either species).

We analyzed the relationship between style length and the proportion of conspecific pollen to heterospecific pollen deposited, which we described as “pollen purity.” Linear and quadratic models were fitted for each species at the two sites. Because there are only two co-occurring *Pelargonium* species at Bosfontein, we expected that for the shorter styled species (*P. pinnatum*), pollen purity will decrease in a linear fashion with increased style length. For the longer styled species (*P. carneum*), we expected an increase in pollen purity with increasing style

length. At Suurbraak, where there are three co-occurring species, we expected that the species with the shortest styles (*P. pinnatum*) will show a negative relationship, and the species with the longest styles (*P. carneum*) would show a positive linear relationship between style length and pollen purity (similar to the Bosfontein population with two species). However, we expected that the species with intermediate style length (*P. dipetalum*) may decrease in pollen purity when its styles are too long or too short and overlap with the style lengths of the other two co-occurring species (i.e., negative quadratic relationship). Because introduced flowers were chosen to increase the stylar overlap between species, it is expected that analyses including the introduced flowers are more likely to demonstrate the trends described. We used generalized linear models with binomial error distributions and logit link functions. The dependent variable was treated as the number of events (conspecific pollen grains received) occurring in a set of trials (conspecific plus heterospecific pollen grains) and style length was treated as the independent variable.

The effect of IPT on seed set: At a single community (Bosfontein), we performed reciprocal crosses between *P. pinnatum* and *P. carneum*. First, we performed reciprocal interspecific crosses between *P. pinnatum* and *P. carneum* using pollen from three different heterospecific donors per cross (*P. pinnatum*: $n = 20$; *P. carneum*: $n = 39$). Next, we simulated IPT by conducting crosses using mixed pollen loads of equal amount. Three recently dehisced anthers (from different donors) from each of the two-co-occurring species were gently rubbed onto the stigma lobes of each pollen recipient. The application of the pollen was alternated so that different donor species were applied first and last to different recipient species (*P. pinnatum*: $n = 19$; *P. carneum*: $n = 46$). For controls, we performed crosses within the same species, again using pollen from three different individuals for each cross (*P. pinnatum*: $n = 20$; *P. carneum*: $n = 33$). Inflorescences were bagged before and after experimental treatments to prevent visits by pollinators.

Seed set was counted after two weeks and fertilized ovules were distinguished from aborted ovules that were much smaller and shriveled in appearance. Seed set differences among conspecific controls, mixed pollen crosses, and interspecific crosses were analyzed for each recipient species using a nonparametric Kruskal-Wallis test with a Dunn-Šidák post hoc test for pairwise comparisons. This analysis was conducted using the R package “PMCMR.”

IS DIVERGENCE GREATER IN SYMPATRY THAN ALLOPATRY?

During nonconsecutive years from March-April 2013 to 2019, *Pelargonium* communities were sampled by measuring the exertion lengths of fully receptive styles from long-tubed

Pelargoniums at 29 localities (16 sympatric communities and 13 allopatric populations; Table S1). Populations that did not co-flower with any other long proboscis fly pollinated *Pelargonium* species were termed “allopatric.” Of the sympatric communities, there were 13 with two species, two with three species, and one with four species. Localities all fell within the known ranges of the two long proboscis fly pollinators, *P. longipennis* and *P. gan- glebauri* (Ethan Newman unpublished data). Below, we outline two tests of the predictions that species should have different style lengths in sympatry but overlapping style lengths in allopatry (see Fig. 2). In the first test, we determined whether there is an overdispersion pattern in style lengths using all the available *Pelargonium* communities. In the second test, we focused on the two most widespread, co-occurring species and specifically tested whether style lengths are more different in sympatry than in allopatry. Other species in these communities were not appropriate to use for this test because they occur at too few localities.

Testing for overdispersion in *Pelargonium* communities

Character displacement is expected to generate a pattern of trait overdispersion in sympatric *Pelargoniums*. More specifically, style length divergence of sympatric species should be greater than expected by chance (i.e., style length differences drawn randomly from the range of observed population style length averages). We explored this expectation by comparing sympatric divergence in style exertion length to randomly assembled null communities using an “evolutionary null model” designed by Muchhala and Potts (2007). If floral variation of guild communities is explained by local evolution in the null model, then this suggests that patterns of trait overdispersion are unlikely due to ecological sorting. The model of Muchhala and Potts (2007) was specifically designed to test for character displacement of stamen/style length exertion in the bat pollinated genus *Burmeistera* and hence its applicability here is highly appropriate.

The evolutionary null model focuses on all *Pelargonium* species occurring in more than one community, namely, *Pelargonium pinnatum* (five communities), *P. carneum* (15 communities), *P. dipetalum* (three communities), *P. myrrhifolium* var. *myrrhifolium* (seven communities), *P. laevigatum*, and *P. gracillimum* (two communities), asking what the expected assemblage structure would be if they evolved local exertion lengths at random. The model randomly permutes the nonzero entries for each column of the widespread species. Hence, the model preserves the observed structure and does not change the position of the blank cells or identity of species within a single locality. For the above model, we generated 1000 null matrices and analyzed their local divergence in the following manner:

Species within a community were first sorted from the shortest to the longest exertion length within each locality. Then, we calculated the mean difference in the exertion lengths of sequen-

tially adjacent species pairs (e.g., at a site with three species, we calculated differences in mean style exertion length between the first and the second, and then between the second and third species). We then calculated the mean style length difference for each matrix (observed and nulls) and generated a frequency distribution of this value for the 1000 null matrices and compared the observed mean differences with this frequency distribution. If fewer than 5% of the null models have greater average style length divergence between sequentially adjacent species pairs than the calculated observed differences, then it suggests significantly greater sympatric style length divergence than predicted by the distribution of style lengths for the species (see Muchhala and Potts 2007).

Focused test on two species

We investigated whether the two most widespread species—*P. carneum* and *P. myrrhifolium* var. *myrrhifolium*—show more exaggerated differences between style lengths in sympatric populations than between allopatric populations. For sympatric populations, we used all six communities in which the two species co-occur and calculated the difference between style lengths (using population means) in each of the six communities. To determine style length differences between these two species for the 12 allopatric populations (four *P. carneum* and eight *P. myrrhifolium* var. *myrrhifolium* populations), we used a null modelling approach to generate four randomized style length comparisons among the species, drawn without replacement from the existing allopatric communities. To do this, differences in style length were obtained by independently permuting the order of populations by “species columns” (1000 iterations), using the function “randomMatrix” in the R package “picante” and subsequently subtracting “species columns.” This provided six differences among sympatric populations and four differences among allopatric populations. We used a one-tailed Independent Samples *t*-test to determine whether the observed style length differences in sympatry were greater than the style length differences of randomized populations generated from observed allopatric style length means. All the above analysis was conducted in R (R Core Team 2017) unless otherwise stated.

Results

STYLE LENGTH SIMILARITY LEADS TO REPRODUCTIVE INTERFERENCE

Stamen and style exertion length and pollen placement

Comparisons of stamens and styles within localities: There are differences in the minimum and maximum anther exertion lengths of *P. pinnatum* and *P. carneum* at Bosfontein (minimum difference: $U = 2.336$, $P < 0.001$; maximum difference:

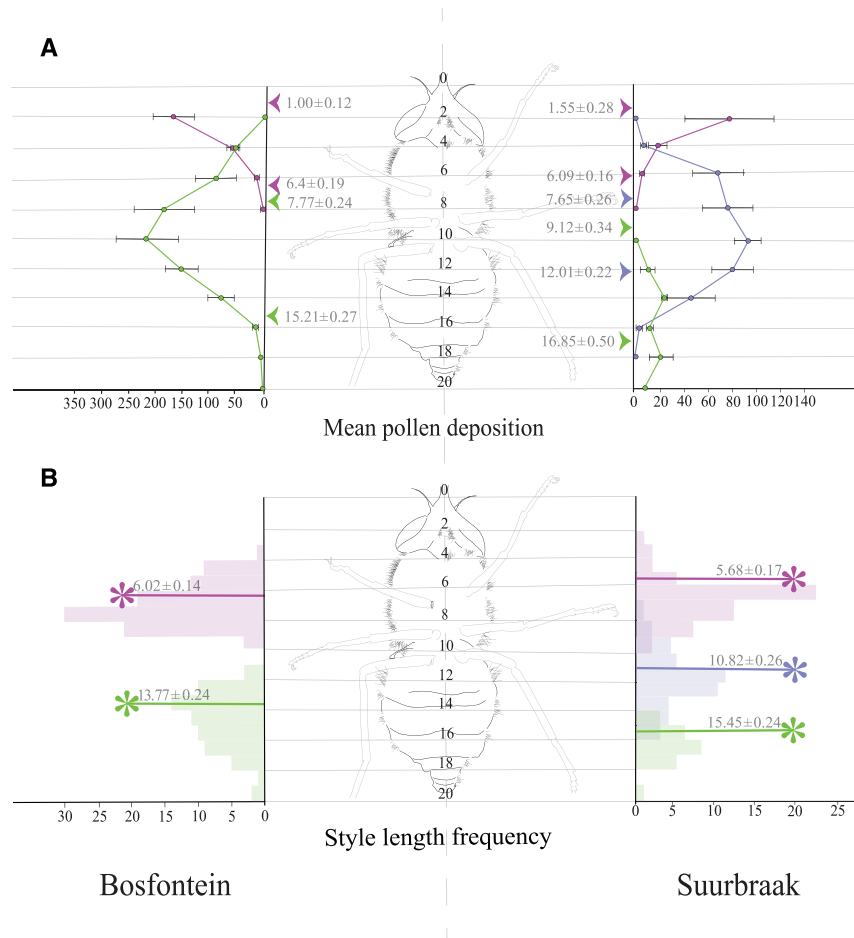


Figure 3. The overlap of stamen and style exertion lengths between co-occurring *Pelargonium* species at two sites and the consequences of this in terms of pollen placement and receipt. (A) At Bosfontein (left), *Pelargonium pinnatum* (purple) and *Pelargonium carneum* (green) differ in their mean minimum and maximum anther exertion lengths with standard errors (depicted by the purple and green arrows under mean pollen deposition), which suggests no overlap in their placement of pollen on fly bodies. This prediction is met with actual counts of mean pollen deposition (\pm standard errors) across 2mm increments, depicted along a standardized fly body. Similarly, at Suurbraak (right), with an additional species *P. dipetalum* (blue), differences in their mean minimum and maximum anther exertion lengths with standard errors are also evident despite overlap of *P. dipetalum* with *P. carneum*. This overlap is also shown in actual pollen placement positions. In addition, the above figure establishes how *Pelargonium* species displace one another for pollen placement position on fly bodies: the presence of *P. dipetalum* at Suurbraak replaces the 10mm region of maximum pollen placement for *P. carneum* at Bosfontein. This displaces *P. carneum* pollen placement further down the bodies of flies at Suurbraak in comparison to Bosfontein. (B) Style exertion length overlaps with the regions of maximum pollen placement at both localities as indicated by style length histograms (mean style length with standard errors is indicated by different color asterisks), suggesting accurate receipt of conspecific pollen by stigmas.

$U = 2.337$, $P < 0.001$; Fig. 3A). This translated into different pollen placement sites on flies by *P. pinnatum* and *P. carneum* ($\chi^2 = 421.466$, $df = 1$, $P < 0.001$; Figs. 2C, 2D, 3A, and S1A). *Pelargonium pinnatum* places most pollen on the head and first few thoracic segments, approximately 1.76 mm from the proboscis (Figs. 2C, 2D, and 3A). In contrast, *P. carneum* places most pollen at the junction between abdomen and thorax, approximately 8.9 mm from the proboscis (Figs. 2C, 2D, and 3A). However, there is a zone on the thorax (4–8 mm from the base of the proboscis) where pollen loads are mixed (see Figs. 2D,

and 3A). While pollen receipt from these areas is likely to result in considerable IPT, style length can potentially influence the chances of contact with these areas. The exertion lengths of *Pelargonium* styles at Bosfontein were not overlapping ($U = 1.755$, $P < 0.001$; Fig. 3B) and appeared to roughly correspond (albeit slightly shifted) to areas of dense and relatively “pure” pollen loads on pollinator bodies (Fig. 3B): *P. pinnatum* styles were very short and are in a good position to receive pollen from underneath the head of the pollinator, whereas the longer styles of *P. carneum* appear more likely to receive pollen from the ventral

thoracic-abdominal junction and mesothorax of a pollinator's body (Fig. 3B).

At Suurbraak, there are three species (not two) competing for pollen placement sites on the ventral parts of long proboscis fly pollinators. The additional species leaves less space available for unique pollen deposition sites and results in some overlap in the range of reproductive parts and pollen placement positions. Minimum anther exertion lengths differed among species ($W = 29.820$, $df = 2$, $P < 0.001$; Fig. 3A), although for one species pair (*P. dipetalum*–*P. carneum*), there was extensive overlap ($P = 0.16$; Table S2). Nevertheless, there are strong statistical differences in maximum anther exertion length ($F = 288.401$, $df = 2$, $P < 0.001$; Fig. 3A), which differed across all pairwise post hoc comparisons ($P < 0.001$; Table S2). This resulted in statistical differences ($\chi^2 = 1788.767$, $df = 1$, $P < 0.001$; Fig. 3A) of mean pollen placement position, where the average distance from the proboscis was 1.42 mm for *P. pinnatum*, 8.76 mm for *P. dipetalum*, and 14.27 mm for *P. carneum* ($P < 0.001$ for all post hoc comparisons; see Figure S1). Similar results were found for style length ($F = 495.56$, $df = 2$, $P < 0.001$; Fig. 3B) where all pairwise post hoc comparisons were also different ($P < 0.001$; Table S2), and roughly matched the areas of peak pollen deposition (Fig. 3B).

Comparisons of stamens and styles between localities: The presence of *P. dipetalum* in the Suurbraak community appears to be associated with a shift in the reproductive parts and pollen placement position of *P. carneum* (Fig. 3B). Specifically, at Suurbraak (Fig. 3B), the minimum and maximum anther exertion lengths are displaced toward the rear of the insect when compared to the Bosfontein population with only two competing *Pelargonium* species (minimum anther exertion length: $t = 2.41$, $df = 67$, $P = 0.019$; maximum anther exertion length: $t = -2.56$, $df = 67$, $P = 0.013$; Fig. 3). Consequently, *P. carneum* differs in the area of pollen placement between localities ($\chi^2 = 14.139$, $df = 1$, $P < 0.001$; Fig. 3). This is matched by differences in style length ($t = -4.522$, $df = 97$, $P < 0.001$; Fig. 3) across the two localities, which match the peak regions of pollen placement by each species (Fig. 3). Anther exertion length was also marginally different between the two *P. pinnatum* localities (minimum anther exertion length: $t = -2.17$, $df = 56$, $P = 0.035$; maximum anther exertion length: $t = 2.58$, $df = 56$, $P = 0.012$; Fig. 3). This marginal difference did not result in detectable differences in pollen placement sites between Bosfontein and Suurbraak ($\chi^2 = 3.029$, $df = 1$, $P = 0.082$; Fig. 3). Nor were there detectable differences in style exertion length between the two localities ($t = 1.53$, $df = 156$, $P = 0.129$; Fig. 3).

Style length overlap and IPT

Pollen purity –style length correlations on natural variation: At Bosfontein, we found a significant negative lin-

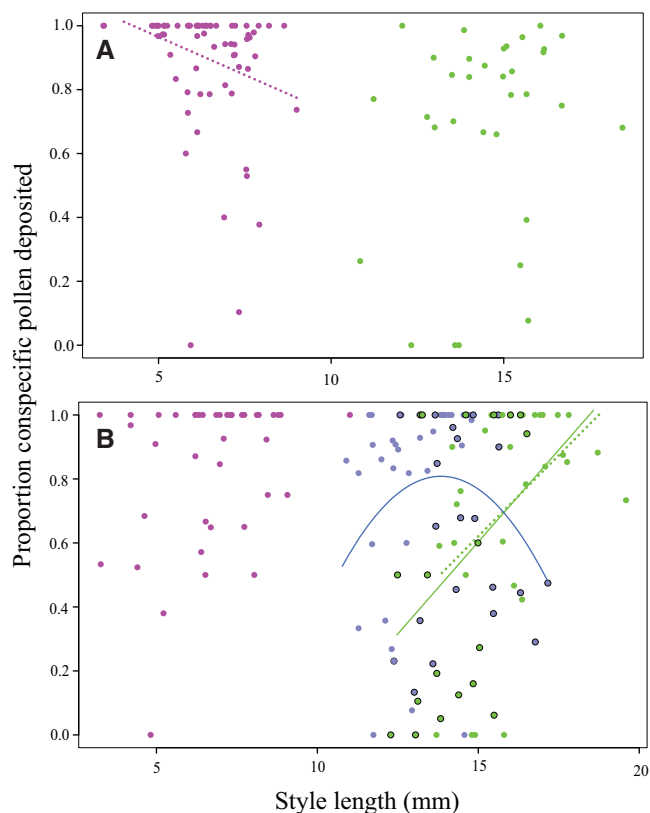


Figure 4. Style length similarity reduces the purity of pollen loads on stigmas. At Bosfontein (A), there is a significant negative linear association between style length and pollen purity for *P. pinnatum* (purple dotted line) and no linear or quadratic relationship for *P. carneum*. At Suurbraak (B), *P. pinnatum* shows no relationship, whereas *P. dipetalum* shows a significant negative quadratic association (blue solid line) when style length variation is increased. Similarly, *P. carneum* also shows a significant positive association between style length and pollen purity (green dotted line), including when style length variation is increased (green solid line). Introduced variation is indicated by circled blue and green data points for *P. dipetalum* and *P. carneum*, respectively.

ear association between style length and the purity of pollen loads for *P. pinnatum* ($Z = -1.91$, $P = 0.050$; Fig. 4A). Pollen purity decreases with increasing style length because longer styles overlap more with the pollen placement sites of *P. carneum*. Quadratic relationships were not significant ($Z = -0.64$, $P = 0.525$; Fig. 4A). *Pelargonium carneum* on the other hand showed no significant linear ($Z = 1.23$, $P = 0.220$; Fig. 4A) or quadratic ($Z = -0.910$, $P = 0.363$; Fig. 4A) association between pollen purity and style length.

At Suurbraak, we found no significant linear nor quadratic associations between style length and the purity of the pollen for *P. pinnatum* ($Z = 1.069$, $P = 0.285$; $Z = 0.484$, $P = 0.629$; Fig. 4B). Nor did we find any significant linear nor quadratic trend for *P. dipetalum* ($Z = 1.724$, $P = 0.085$; $Z = 0.662$,

$P = 0.508$; Fig. 4B). We detected a positive linear association between *P. carneum* pollen purity and style length ($Z = 3.150$, $P = 0.001$; Fig. 4B) but no quadratic association ($Z = -1.641$, $P = 0.100$; Fig. 4B). This suggests that the purest pollen loads are on *P. carneum* styles that are most different in length from the styles of *P. dipetalum*.

Pollen purity-style length correlations with introduced variation: When individuals of *P. dipetalum* and *P. carneum* were introduced from outside populations to create greater style length overlap between the coexisting species, we found a significant negative quadratic association between the purity of pollen and style length of *P. dipetalum* ($Z = -2.243$, $P = 0.025$; Fig. 4B). This suggests that the pollen purity of *P. dipetalum* decreases as its style lengths extend into the ranges of the two co-occurring *Pelargonium* species. No significant linear relationship between style length and pollen purity was detected for this species after the addition of phenotypic variation from outside populations ($Z = -1.364$, $P = 0.173$; Fig. 4B). The introduction of phenotypic variation resulted in a positive relationship between *P. carneum* style length and pollen purity. This was similar to the relationship observed in the natural population, although the added replication at the extremes increased the robustness of the association ($Z = 5.778$, $P = 0.001$; Fig. 4B). The quadratic relationship was nonsignificant ($Z = -0.586$, $P = 0.558$; Fig. 4B).

The effect of IPT on seed set: Differences in seed production after reciprocal crosses using mixed versus conspecific pollen loads at Bosfontein were significant for both *P. pinnatum* ($\chi^2 = 25.81$, $df = 2$, $P < 0.001$) and *P. carneum* ($\chi^2 = 61.21$, $df = 2$, $P < 0.001$) recipients. Mixed pollen loads (*P. pinnatum* + *P. carneum*) to *P. pinnatum* recipients resulted in marginally fewer seeds than pure *P. pinnatum* pollen loads ($P = 0.060$; Fig. 5). Application of interspecific pollen loads resulted in no seed set for either species, which was significantly fewer seeds than produced by pure intraspecific crosses ($P = 0.001$; Fig. 5).

Similar results were found for *P. carneum*: Mixed pollen loads to *P. carneum* recipients resulted in significantly lower seed set when compared to pure *P. carneum* crosses ($P = 0.001$; Fig. 5). Similarly, no seeds were produced after heterospecific crosses using *P. pinnatum* donors to *P. carneum* recipients, and this finding was also significant when compared to pure *P. carneum* crosses ($P = 0.001$; Fig. 5).

OVERDISPERSION PATTERN IS CONSISTENT WITH CHARACTER DISPLACEMENT

Overdispersion in Pelargonium communities

Patterns of trait overdispersion appear evident in the communities with little trait overlap among species within sites but frequent overlap when those same species occur in allopatry (Fig. 6A). A formal test supported these visual observations: On

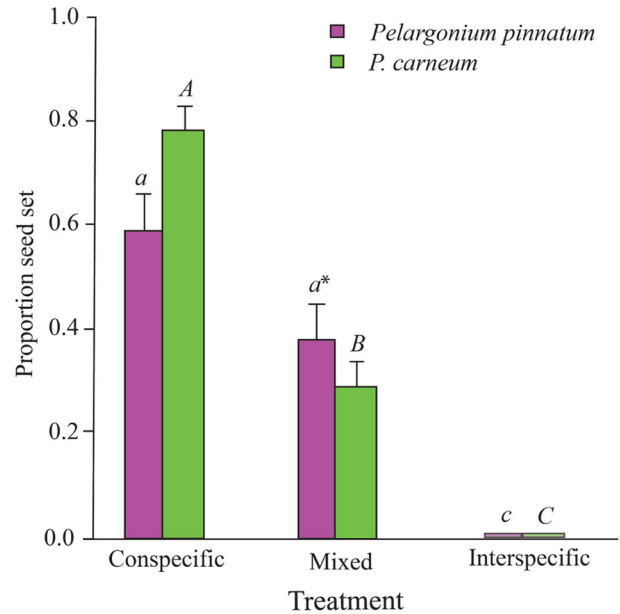


Figure 5. Reciprocal crosses between *P. pinnatum* and *P. carneum* at Bosfontein. Crosses with mixed pollen loads sire fewer seeds than crosses using donors with pure conspecific pollen (*P. pinnatum*, $P = 0.060$; *P. carneum*, $P = 0.001$). Heterospecific pollen donors sired no seeds (*P. pinnatum*, $P = 0.001$; *P. carneum*, $P = 0.001$). Significant differences among treatments are indicated by different letters a, b, and c in lowercase for *P. pinnatum* and uppercase for *P. carneum*. Asterisk indicates borderline significance at $P = 0.06$.

average, the observed style length differences in sympatry were 5.93 ± 0.56 mm (mean \pm SE). In contrast, the expected mean style exertion length differences of communities generated by the evolutionary null model were smaller 5.37 ± 0.01 mm (mean \pm SE). Of the 1000 generated null community matrices for style length, only six had greater mean style length exertion differences than the observed communities (i.e., $P = 0.006$).

Focused test on two Pelargonium species

On average, the style length difference between sympatric populations of *P. carneum* and *P. myrrhifolium* var. *myrrhifolium* was 4.07 ± 0.50 mm (mean \pm SE; Fig. 6B), whereas the style length difference using randomized comparisons between allopatric populations of the same two species was 1.935 ± 0.84 mm (mean \pm SE; Fig. 6B). This suggests that for these two wide ranging species, style length differences tend to be larger in sympatry than in allopatry ($t = -11.72$; $df = 3$; $P = 0.001$; Fig. 6B).

Discussion

Several lines of evidence demonstrate processes and patterns consistent with character displacement. Specifically, we found that similarity in floral reproductive parts results in pollen being placed

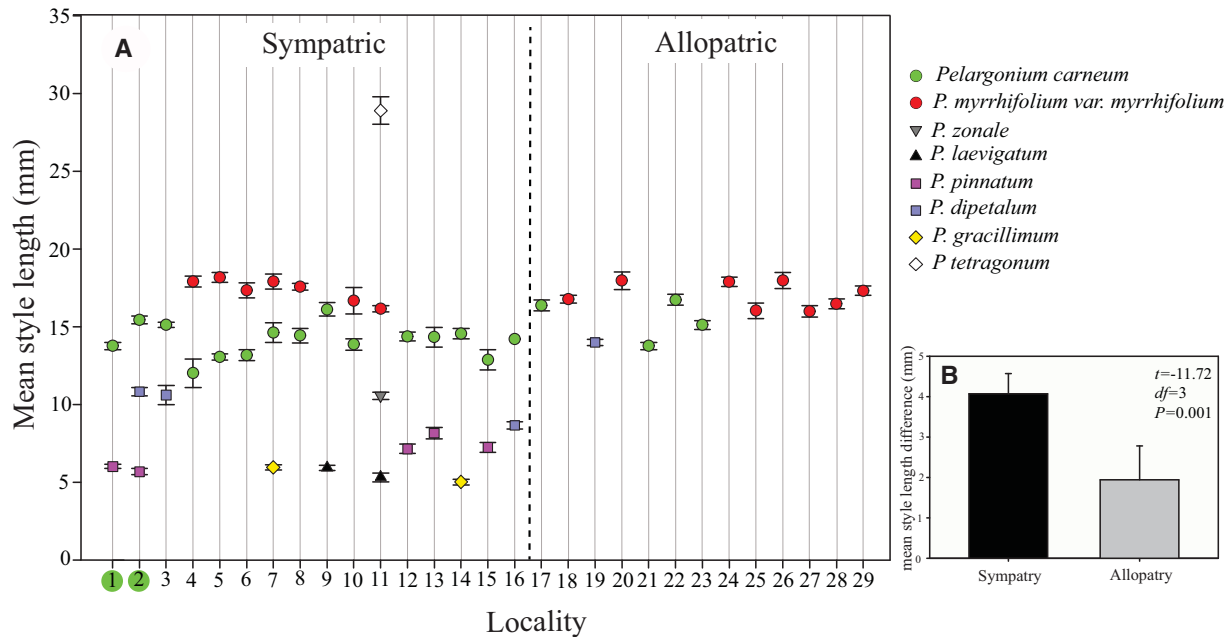


Figure 6. Style length similarity in sympatric versus allopatric populations. (A) Mean style exertion lengths with standard errors for eight *Pelargonium* species across 29 localities. Localities 1 and 2 in green circles indicate Bosfontein and Suurbraak experimental sites. In the above figure, sympatric communities have highly divergent style exertions that never overlap (locality: 1–16), a pattern clearly demonstrated in the two most widespread species highlighted in green (*Pelargonium carneum*) and red (*P. myrrhifolium* var. *myrrhifolium*). However, in allopatry (locality: 17–29), these same widespread species can have overlapping style exertion lengths. (B) This leads to statistically greater style exertion lengths among sympatric red and green communities than among allopatric red and green populations.

on the same areas of pollinator bodies and that this results in IPT. We then demonstrate that IPT has negative consequences on plant fitness as it reduces seed production. These negative interactions between co-occurring *Pelargonium*s provide a potential mechanism for character displacement. Supportive evidence that negative interactions resulted in character displacement was demonstrated through an overdispersion pattern where *Pelargonium* species always have large style length differences in sympatry, but often overlapping style lengths in allopatry. This pattern is replicated across multiple populations and among several species and has been formally tested in the two most widespread species. Our study provides a potential link between mechanistic process and overdispersion pattern in these plant communities and suggests that character displacement has driven phenotypic divergence. From process to pattern, we discuss the evolution of character displacement in this study system and whether it likely evolved through resource competition or reproductive interference.

STYLE LENGTH SIMILARITY LEADS TO REPRODUCTIVE INTERFERENCE

Stamen and style exertion length and pollen placement

Our study provides rare evidence of shifts in pollen placement matched by shifts in style length, which have the potential to re-

duce reproductive interference. Analyzing pollen placement sites on pollinators is often not possible for closely related species (Minnaar et al. 2019a,b), although in this case, unique pollen color and shape characters have allowed us to present this aspect of the study (c.f. Muchhala and Potts 2007 who use pollen color to distinguish pollen on stigmas but not on pollinators). Specifically, *P. carneum* utilizes pollinator bodies in slightly different ways at two sites (Bosfontein and Suurbraak) and this difference appears to be associated with changes in co-occurring *Pelargonium* communities. In particular, an additional species (*P. dipetalum*) at the Suurbraak site is associated with a shift in the reproductive parts of *P. carneum* (relative to their positions at the Bosfontein site where *P. dipetalum* is absent). *Pelargonium carneum* reproductive parts were longer in the presence of *P. dipetalum* so that they placed and received pollen on the abdomen of the pollinator instead of the junction between the thorax and abdomen. Similar shifts in phenotype were not observed for *P. pinnatum* because it is difficult for the reproductive parts of this species to be any shorter than they already are. Because pollinators can be viewed as resources (Pauw 2012), changes in exertion and pollen placement by *P. carneum* suggest that a shift in floral morphology changes the way that flowers use the pollinator resource, an important criterion for demonstrating character displacement (Schluter and McPhail 1992). Furthermore, if changes in pollen

placement reduce pollen overlap on the body of a pollinator (as our results demonstrate), it may also reduce IPT and reproductive interference (Moreira-Hernández and Muchhala 2019).

Style length similarity increases IPT

In natural populations, style-length-mediated IPT may be difficult to detect if strong selection has already reduced reproductive interference by eliminating most individuals with overlapping phenotypes (Schluter 1988; Germain et al. 2018). This may explain why natural stylar exertion was not associated with pollen purity for *P. carneum* at Bosfontein and *P. pinnatum* and *P. dipetalum* at Suurbraak (Fig. 4). Furthermore, pollen movement between style length phenotypes is often asymmetric and hence not all interacting species will experience equal levels of IPT (Minnaar et al. 2019b; Wolf et al. 2001; Kay 2006). We found the strongest evidence for style-length-mediated IPT between species by increasing the style length variation within a population after introducing individuals from outside populations. These introduced individuals had style exertions that were more similar in length to other local *Pelargonium* species and consequently received more heterospecific pollen. In particular, a quadratic relationship between pollen purity and style length was found after increasing style length variation of *P. dipetalum*. We interpreted this as *P. dipetalum* individuals with extremely short or long styles receiving more heterospecific pollen from *P. pinnatum* or *P. carneum*, respectively (Fig. 4B). Muchhala and Potts (2007) also found that style length divergence was associated with pollen purity in bat pollinated *Burmeistera* (Campanulaceae). These results are consistent with the sexual architecture hypothesis that predicts a reduction in IPT with reproductive-part divergence of co-occurring species (Murcia and Feinsinger 1996), thereby reducing reproductive interference and increasing fitness (Dressler 1968; Brown and Kodric-Brown 1979; Muchhala and Potts 2007; Muchhala and Thomson 2012). Overall, our findings suggest that the limited pollen-placement space on the pollinator results in reproductive interference between co-occurring *Pelargonium*s. However, this interference is most intense when heterospecific individuals have similar style lengths.

IPT reduces seed set

If character displacement is the driver of shifts in style length morphology, then style length differences need to be linked to plant fitness. We establish this link by showing greater IPT with increasing style length similarity of co-occurring *Pelargonium* species (Fig. 4). In addition, we also demonstrate that IPT has negative consequences for female fitness because mixed pollen loads lead to reduced seed set in both species at Bosfontein (Fig. 5). Because *Pelargonium*s only produce five seeds per flower, reduced seed set in these experiments is unlikely to be the consequence of conspecific pollen limitation but rather a negative effect of

the heterospecific pollen. Although we did not demonstrate the negative effects of IPT for all of the co-occurring *Pelargonium* species in our study, these results contribute to a much larger body of literature showing that IPT commonly has negative effects on female fitness (Fishman and Wyatt 1999; Wilcock and Neiland 2002; Gomez and Ashman 2014; Moreira-Hernández and Muchhala 2019; Moreira-Hernandez et al. 2019). Although not explicitly investigated in our study, IPT should also have automatic male fitness implications: when pollen lands on the stigma of another incompatible species, it is effectively lost because it can no longer be used for fertilization (Moreira-Hernández and Muchhala 2019). Consequently, IPT among sympatric *Pelargonium* species in Bosfontein is expected to affect male fitness negatively because *Pelargonium*s are not cross compatible (Minnaar et al. 2019a; Muchhala and Thomson 2012). Taken together with the correlations between style length and IPT, these results suggest that style length similarity has negative fitness consequences for co-occurring species and that this has the potential to select for phenotypic divergence in sympatry (Moreira-Hernández and Muchhala 2019).

OVERDISPERSION PATTERN IS CONSISTENT WITH CHARACTER DISPLACEMENT

Across the 29 *Pelargonium* communities, we found a strong visual pattern of stylar overdispersion consistent with character displacement (Fig. 6): This pattern was supported by our two null model comparisons, one using all the species that occur at more than one site and another exploring the relationship between just the two most commonly co-occurring species. The model using all widespread species was highly constrained so that ecological origins of the pattern were excluded, suggesting an evolutionary origin of the pattern. Using the same analysis, Muchhala and Potts (2007) found equivocal evidence for their evolutionary null model ($P = 0.080$). Nevertheless, the evolutionary component for character displacement was supported by flight cage experiments that demonstrated higher IPT among species with similar styles. Armbruster et al. (1994) also used a similar model to exclude the possibility that ecological sorting was the driving force behind patterns of trait overdispersion and found that local evolutionary shifts were responsible for shaping phenotypic variation within Australian trigger plant communities (*Stylidium*). Our results suggest that *Pelargonium* style length shifts associated with community composition have occurred on multiple occasions, across multiple species, and over multiple sites and that the pattern is unlikely the result of chance.

Although our results suggest that IPT is the likely driver of these patterns, it is also important to consider other possible factors. Notably, abiotic variation may potentially generate geographic trait variation (Lambrecht and Dawson 2007). However, populations that show style length shifts are often in very close

proximity (<5 km), and they often occur on the same soil types and experience similar rain fall. Consequently, it seems implausible that variation in the abiotic environment will consistently cause the divergence of the same phenotypic trait in closely related, sympatric plants. More likely, one would expect that traits such as style length would shift in the same direction (not opposite directions) in response to a common environmental variable.

RCD VERSUS ECD

Character displacement can occur as a result of resource competition (ECD) or reproductive interference (RCD). However, these two mechanisms need not be mutually exclusive and frequently one may facilitate the other (reviewed by Pfennig and Pfennig 2010). For example, divergence of bird beak and larynx morphology in response to limited food resources can affect traits involved in species recognition such as mating calls (Grant and Grant 2011). The most commonly identified direction of influence is when phenotypic shifts related to resource use affect reproductive traits (Podos and Nowicki 2004; Price 2008). However, in theory it is also possible that the direction of influence can be reversed: where divergence to limit reproductive interference can cause shifts in resource use (Konuma and Chiba 2007).

Character displacement among *Pelargonium*s in this study appears to have elements of both ecological character displacement and RCD, and it may not be obvious how and whether one mechanism is driving the other. In this manuscript, we have suggested that RCD drives divergence so that reproductive interference between co-occurring *Pelargonium*s is reduced. However, it could also be said that pollinators are a resource and that co-occurring plants compete for pollen placement sites on the body of a shared pollinator. Here, plants appear to divide the body of the pollinator into different niches, so that within a site, different species specialize by placing pollen on different parts of the pollinator and in so doing, they alleviate competition for the pollinator resource. Similarly, *Anolis* lizards specialize and utilize different parts of trees to reduce competition (Losos 2009), suggesting that in both systems, resource scarcity may be driving divergence. However, despite the parallels between pollinator resource partitioning by *Pelargonium*s and tree resource partitioning by *Anolis* lizards, the underlying reason for *Pelargonium*s to partition the pollinator niche appears to be the reduction of reproductive interference. Consequently, we argue that this is a case of RCD driving shifts in the way that *Pelargonium*s use resources rather than the other way around.

RCD is often thought to play a special role in the speciation process of plants and animals because it sharpens species boundaries by increasing reproductive isolation (Pfennig and Pfennig 2012). In the case of plants, this study demonstrates that RCD of anther and style morphology causes differences in

the positions of pollen placement and receipt sites on pollinators. This is known to generate assortative mating patterns and contribute toward reproductive isolation (Minnaar et al. 2019b; Armbruster et al. 2014). More specifically, the fact that reproductive isolation can be directly caused by phenotypic divergence of style and anther exertion (Minnaar et al. 2019b) suggests that RCD has the potential to drive ecological speciation in plants.

Conclusion

Previous studies on the *Prosoeca longipennis-ganglebauri* long-proboscid fly pollination guild suggest that floral variation, specifically tube length, has evolved in response to variation in pollinator morphology (Anderson et al. 2014; Newman et al. 2014; Newman et al. 2015). In this study, we highlight a nonpollinator agent of selection (variation in plant community structure), which appears to drive the evolution of “community ecotypes.” In this manuscript, the reproductive parts of *Pelargonium* flowers appear to be adapted to different *Pelargonium* communities. Traditionally, the study of floral evolution has centered on pollinators, with other drivers such as seed dispersal, competition, or herbivory receiving considerably less attention (Strauss and Whitall 2006). These results provide an interesting contrast because they demonstrate that in addition to pollinators, the interactions between plants and other floral community members are potentially also important drivers of floral evolution and speciation. In this particular case, plant community members interact with one another via pollinators, highlighting a need to study the various drivers of floral variation in concert (Ramos and Schiestl 2019), rather than in isolation.

AUTHOR CONTRIBUTIONS

BA and EN designed the research. EN collated the data and performed all analyses. EN wrote the first draft. EN and BA wrote the paper.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA ARCHIVING

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Supporting Information

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

Table S1. Mean style length exertions with standard errors and population sizes of twenty-nine *Pelargonium* communities across the Western and Eastern Cape of South Africa.

Table S2. Pairwise comparisons of reproductive traits from *post hoc* tests in one-way ANOVA and Kruskal-Wallis analysis at Suurbaak.

Figure S1. Back transformed means and resultant asymmetric error bars for pollen placement sites of *Pelargonium* species on the bodies of flies' that occur at a locality with two species A) Bosfontein, and a locality with three species B) Suurbaak.