

Structural anther mimics improve reproductive success through dishonest signaling that enhances both attraction and the morphological fit of pollinators with flowers

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Numerous studies have identified traits associated with anther mimicry; however, the processes underlying floral deception remain poorly documented for these structures. We studied the importance of pollinator attraction and mechanical fit of anther mimics in *Tritonia laxifolia* (Iridaceae) and their relative contributions to reproductive success. To determine anther mimics role in attraction, we offered bees' binary choices to flowers painted with UV-absorbent and UV-reflecting paints. We also conducted preference experiments between flowers with excised anther mimics and unmanipulated controls, from which mechanical fit was assessed by allowing single visits. Anther mimics' effects on female reproductive success were determined using similar treatments, but on rooted plants. Bees preferred UV-absorbent over UV-reflecting anther mimics. Bees did not discriminate between flowers with and without three-dimensional anther mimics. Single visits resulted in more pollen deposition on unmanipulated controls over flowers with their anther mimics excised, which was directly linked to pollen-collecting behavior. Controls with unmanipulated anther mimics had higher seed set than those with their anther mimics excised. This study provides insights into pollinator-mediated selection on deceptive floral signals and shows that three-dimensional anther mimics increases reproductive success through both attraction and pollen-collecting behaviors.

KEY WORDS: Color perception, morphological fit, pollen deposition, pollen mimicry, pollen-collecting behavior, seed set, preferences, *Tritonia laxifolia*.

Mimicry, the phenomena in which an organism obtains a fitness advantage by resembling the phenotype of the another, provides some of the finest examples of natural selection (reviewed in Anderson and de Jager [2020] and Jamie [2017]). In the most common scenario where a receiver is disadvantaged by the mimic (Newman et al. 2012; Cohen et al. 2021), antagonistic interactions may result in the receiver evolving an increasing ability to discriminate dishonest signals as advertised by the mimic from the model. In response, mimics may evolve phenotypes more similar to that of the models, making it increasingly difficult for receivers to recognize dishonest

signals (Spottiswoode and Stevens 2011). This reciprocal selection exerted by mimics, models, and receivers may result in a coevolutionary arms race (Dawkins and Krebs 1979; Thompson 2005, 2009), wherein trait escalation can result in premating isolation barriers that may drive speciation events (e.g., Jamie et al. [2020]). Under coevolutionary theory, it is expected that the further the mimics phenotype is that from the models, the lower fitness the mimics should experience (Leimar et al. 2012), a finding that is often demonstrated by studies manipulating the phenotype of mimics to test whether their signal is adaptive (Johnson et al. 2003a; Harper Jr. and Pfennig

2007). Despite this finding, imperfect mimicry is a common occurrence and dictates that the mimic does not necessarily need to converge on the phenotype of the model precisely, as long as the receiver is deceived by the mimic's signal (see Kikuchi and Pfennig [2013] for the “*eye of the beholder hypothesis*”).

Furthermore, mimics may deceive a receiver by converging on more than one signal of the model, often exploiting multiple sensory modalities. This could mean that the sum of “imperfect stimuli” signaled to the receiver might make it more difficult to discriminate between mimics and models (e.g., *sensory overload hypothesis*), compared to when they act in isolation (reviewed in Dalziel and Welbergen 2016). For example, Jamie et al. (2020) successfully demonstrated mimicry between *Vidua* finches and grassfinch nestlings, despite differences in the resemblances of patterns, colors, vocalizations, and postural behaviors. Similarly, orchids attract male wasps using floral chemistry that resembles the pheromones of females, despite the morphology of the flowers not precisely matching the bodies of female wasps (Benitez-Vieyra et al. 2009). Therefore, to understand the evolution of mimicry, one needs to disentangle the fitness contributions of different mimetic signals with reference to how it affects the receivers' responses or behaviors.

Many angiosperms exploit the perceptual biases of food-seeking visitors to obtain pollination services through traits involved in attraction, such as flower color (Koski 2020) and scent (Raguso 2008). In return, pollinators receive a nutritional reward, with nectar being the most obvious. However, pollen is often overlooked as a reward but is essential for both solitary and social bees as provisions for their larvae and energy requirements. Because pollen foraging reduces male fitness, to compensate, many plants have evolved floral signaling structures that imitate pollen (referred to here as pollen-imitating structures) and deceive insects into visiting flowers to forage on pollen, but it improves the reproductive success of the plant instead (Vogel 1975; Osche 1979, 1983a,b; Lunau 2006). These pollen-imitating structures often share UV-absorbent colors between 500 and 600 nm that resemble the bright yellow flavonoids and carotenoids in the pollenkitt (Harborne and Grayer 1993). Regardless of sharing a similar color signal with pollen, they may also serve as nectar guides to attract nectar foraging insects.

Despite their prevalence (Lunau 2000), the selective advantage of pollen-imitating structures remains understudied. Studies investigating the occurrence of these structures primarily document their presence or spectral reflectance properties and few investigate the underlying evolutionary processes. To date, studies have focused on the functional role of vestigial staminodes (Walker-Larsen and Harder 2001b), including their role in attracting pollinators and improving the morphological fit of pollinators when visiting flowers (Walker-Larsen and Harder 2001b; Dieringer and Cabrera 2002; Guimarães et al. 2008;

Milet-Pinheiro and Schlindwein 2009). These studies compare components of fitness between flowers with their staminodes excised against unmanipulated controls, often considering handling time as an additional fitness surrogate. However, it remains unclear whether improved fitness on unmanipulated controls is the result of the three-dimensional structure of the staminode creating a hindrance to pollinators accessing the nectar reward (Martos et al. 2015), or whether reproductive success is improved when pollen-collecting behavior is focused on the staminode.

Demonstrating the inability of the pollinator (signal receiver) to discriminate between the signals of the model and the mimic is a crucial line of evidence for inferring floral mimicry (Newman et al. 2012; Schiestl and Johnson 2013), including pollen or anther mimicry (Lunau 2000). Although preferences for pollen-imitating structures have been documented (e.g., Milet-Pinheiro and Schlindwein 2009; Lunau 2014; Duffy and Johnson 2015), the lack of evidence for pollen-collecting behavior by bees on pollen-imitating structures remain surprising. This is because pollen collection is an essential behavior of both social and solitary bees, both of which require pollen as nutritional resources for their brood, and such pollen-imitating structures are likely adaptations to exploit such behavior. Nevertheless, Vogel (1978) observed differences in the way that bees handle pollen-imitating structures over stamens. His observations indicate that bees search for pollen using specific behaviors that they do not exhibit when interacting with pollen-imitating structures. However, it is not determined whether it is experienced bees that exhibit these behaviors or not, leaving the question open on whether bees exhibit “true” pollen-collecting behavior on pollen-imitating structures (see Lunau et al. 2017). It is also unknown whether pollen-collecting behavior occurs on both three-dimensional pollen-imitating structures (anther mimics) and two-dimensional pollen-imitating structures (yellow markings).

Tritonia (Iridaceae) is a small African genus of 30 species with pollen-imitating structures ranging from those species having no pollen-imitating structures and “unicolor” tepals to two-dimensional pollen-imitating markings contrasting against the tepal colors, and three-dimensional pollen-imitating structures formed by raised structures projecting from the tepals (referred to throughout this study as anther mimics) (Fig. S1). *Tritonia laxifolia* Bentham ex Baker (Iridaceae) is one such species with prominent anther mimics on its lower lateral and median tepals that appear as yellow teeth-like structures (Fig. 1). Using color vision analysis combined with binary preferences, single visits, and video recordings, we explore multimodal mimicry in this system by studying both the visual (preferences) and tactile responses (pollen-collecting behavior) of pollinators on three-dimensional anther mimics, and the

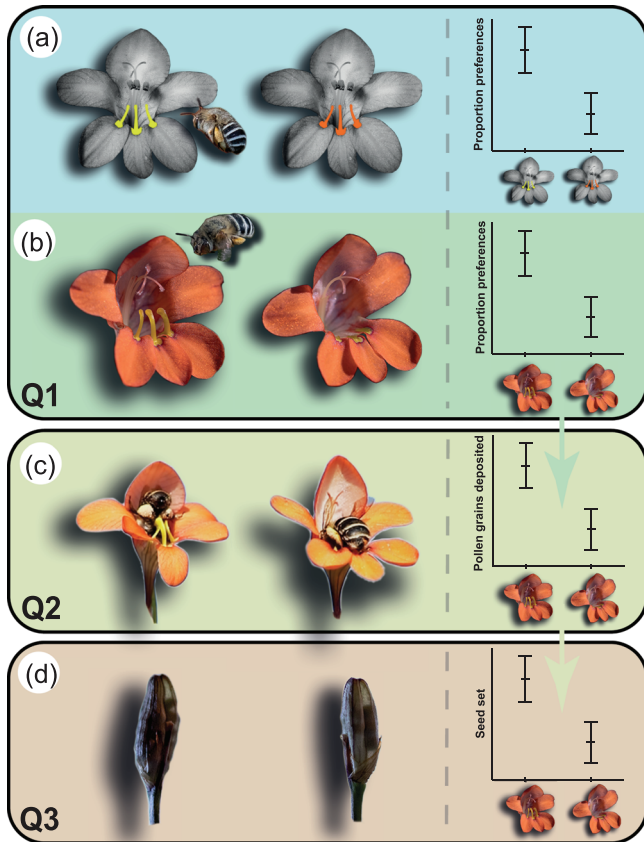


Figure 1. Experimental design and hypothetical outcome of results. Enclosed boxes refer to different research questions related to multimodal anther mimicry. (Q1: Preferences). (a) Pollinators are predicted to prefer UV absorbent yellow paint that is similar in coloration to that of the anther mimics, compared to UV-reflecting orange paint similar to the color of the flower tepals. (b) We predict that pollinators are more attracted to the pronounced signal of the intact three-dimensional anther mimics, compared to the excised structure, leaving behind a reduced two-dimensional colour signal. (Q2 and Q3: Pollen-collecting behavior). (c) More pollen deposition is expected on intact anther mimics compared to those that have been excised (notice the lack of contact with stigma on flowers with anther mimics excised versus retained), and this, together with preferences in (b), should lead to increased seed set in selection experiments using the same experimental approach (d), as indicated by arrows between boxes.

consequences of those responses on fitness. We ask the following questions. (1) Do pollinators prefer the color and structure of anther mimics? (2) Do anther mimics facilitate the morphological fit of pollinators with flowers, and is this associated with pollen-collecting behavior? (3) Do preferences and morphological fit of pollinators to flowers with anther mimics have consequences for female reproductive success? (See Fig. 1 for graphical explanation).

Materials and Methods

STUDY SPECIES AND LOCALITIES

Tritonia laxifolia (Iridaceae) Bentham ex Baker is a small, deciduous, winter-growing geophyte that flowers from March to June in disturbed habitats along the east coast of Africa, from Port Elizabeth in the Eastern Cape of South Africa to Tanzania (de Vos 1982). The scentless, zygomorphic flowers are orange red with the adaxial surface of the dorsal tepal being a contrasting pale pink. The most striking feature of the flowers is the three peculiar bright yellow anther mimics on each of the lower tepals (Fig. 2a,b). In addition, *T. laxifolia* has three inconspicuous, light pink-colored anthers. Receptive stigmas are deeply divided with three style branches becoming recurved and coarsely pustulate when receptive (Manning et al. 2002). Flowers typically last between two and three days and are protandrous with distinct male and female phases (Authors, pers. obs.).

Our study was conducted from April to June in 2019 and 2021, near Fish River Pass, Pikoli (-33.241132° , 27.014889°), (Fig. S2a) and Mosslands farm 18 km southwest of Makhanda/Grahamstown (-33.401357° , 26.432470°) in the Eastern Cape of South Africa. At these localities, *T. laxifolia* occurs on seasonally wet clay in disturbed thicket vegetation dominated by *Euphorbia tetragona*, *Euphorbia triangularis*, and *Aloe ferox*. At both study sites, *T. laxifolia* is primarily visited by *Amegilla fallax* (Fig. 2b,c), with *Apis mellifera scutellata* and pollen-collecting bees present in lower abundance. Medium and large butterflies *Colotis eris eris*, *Pinacopteryx eriphia eriphia*, and *Papilio demodocus* were abundant and frequently visited the flowers (Fig. S2b,c).

DO POLLINATORS PREFER THE COLOR AND STRUCTURE OF ANTHR MIMICS?

Color preferences

To determine if the yellow UV-absorbent color of anther mimics is important in attraction, we conducted binary preferences at Mosslands between the 7th and 23rd of May 2021, between 9:00 a.m. and 2:00 p.m. at temperatures consistently exceeding 20°C . Fresh flowers were picked from the field before pollinators arrived. We removed the yellow UV-absorbent color signal from one-half of the flowers by painting the anther mimics with orange UV-reflecting paint (Dala Neon Orange) that is similar in coloration to the adjacent tepals. We mimicked the yellow UV-absorbent color of the anther mimics by painting the anther mimics of the other half of the experimental flowers with UV-absorbent yellow paint (Dala craft paint, yellow). Paints were applied to the entire UV-absorbent yellow part of the anther mimic using a #7 insect pin at least an hour before preference experiments started and allowed to dry. To control for



Figure 2. Color plate of the study system. (a) *Tritonia laxifolia* (Iridaceae) in flower at Fish River Pass, Pikoli. (b) *Amegilla fallax* approaches a flower, the white arrow highlights three-dimensional anther mimics on each lower tepal. (c) Bee visitor required to crawl onto and over the anther mimics to contact reproductive parts of the flower. (d) UV images of an unpainted control (left) and a flower with its anther mimics painted with UV-reflecting orange paint.

the potential influence of the scent of the paints, an equal amount of paint from the opposite treatment was applied to the inner part of the container serving as a vase to hold the inflorescences. Binary color preferences included two experimental trials: (1) Anther mimics painted with yellow UV-absorbent paint versus orange UV-reflecting paint; (2) Anther mimics painted with yellow UV-absorbent paint versus unpainted anther mimics. Experiment 1 tests the importance of the UV absorbent yellow signal in attracting the pollinator. Experiment 2 is a control that assesses whether the pollinators are equally attracted to the yellow UV-absorbent paint applied in experiment 1 and naturally yellow unpainted anther mimics.

We used the bee interview technique for both experiments (e.g., Johnson et al. 2003b), as the flowers were too numerous within the population to wait for bees to approach stationary arrays. In these experiments, flower pairs were suspended in two 25-mL tubes filled with water and fixed at the end of a bamboo stick (± 2 m) arranged approximately 10 cm apart. Preferences were executed by placing pairs near a foraging pollinator, and pollinators were offered a binary preference. We recorded the insect species and the individual pollinators first preference.

Statistical differences within experimental treatments were determined using generalized linear mixed-effects models (GLMMs) with binomial error distributions and logit link functions, where treatments were assigned as fixed factors and binary preferences as the response. Because it was challenging to swap inflorescences within a pair after each visit to account for nonindependent positioning of pairs, specific pairs (i.e., different preference sticks containing choices) were incorporated as a random factor.

Preference for the physical structure of the anther mimics

We picked a total of 128 inflorescences in bud or the early stages of flowering over 17 days between the 20th of May and the 6th of June 2019. As inflorescences matured, we emasculated flowers using a surgical blade to prevent pollinator preferences from being influenced by flowers in different stages of anthesis (e.g., pollinators may prefer male-phase flowers containing pollen over female-phase flowers). To determine the effects of anther mimics on pollinator preference, we carefully excised all three anther mimics from all available flowers from exactly half of the

experimental inflorescences ($n = 64$) using a surgical blade. The other half remained unmanipulated (only the anthers were removed) ($n = 64$). We refer to these inflorescences/flowers as “anther mimics excised” and “unmanipulated controls” throughout the manuscript (Fig. S2d). Essentially, the excision of the anther mimic removes the physical structure, but the round yellow mark on the tepal that remains after excision serves as a two-dimensional visual component of the anther mimic. Furthermore, physical damage that may influence bee behavior on flowers is accounted for by the removal of the anthers in both treatments (in the same way that anther mimics were removed). These inflorescences were kept in a cool room at 10°C until the stigmas became receptive. Once receptive, experimental inflorescences were used to disentangle the function of the three-dimensional structure of the anther mimic, using experiments that simultaneously test the roles of visual signaling and morphological fit (pollen deposition) in the pollination process, as explained below (also see Fig. 1).

To test whether the physical structure of anther mimics is associated with pollinator attraction, we conducted preference experiments over four days between the 29th of May and 4th of June 2019 between 9:00 a.m. and 2:00 p.m. depending on pollinator activity. Inflorescences with anther mimics excised and unmanipulated controls were organized into 10 pairs with individuals placed approximately 10 cm apart and spaced about 25 cm from other pairs. These pairs were arranged at the same height relative to naturally occurring flowering plants within the population, and the control and experimental inflorescences were matched for the number of open flowers (either one or two). Once a visitor entered the arena, one of the authors recorded the binary preference made by visitors to treatments within a pair. Observers also recorded the sequence of visits to pairs by each pollinator individual. Only first choices were included in the statistical analysis (switches to alternative phenotypes within a pair were excluded from the preference analysis and only used in the single-visit experiments). All pollinators were incorporated in the data analysis. We treated binary preferences as the response and treatments as fixed factors in a GLMM that considered a binomial error distribution and a logit link function, with pollinator individual treated as a random factor to account for nonindependence in the data resulting from repeated visits from the same individual (i.e., controlling for individual behavior).

To determine whether bees can perceive differences between the anther mimics, anthers, pollen, and adjacent flower tissue including paints used in preference experiments, we measured colors from different segments of the flower involved in attraction from between five and seven receptive female-phase flowers from different individuals from Makhanda. We separated anther mimics from the anthers, pollen, dorsal, and lateral sepals using a surgical blade. We also painted the central anther mimics of

three individuals with UV-reflecting orange paint and three individuals with UV-absorbent yellow paint used in color preferences. Once dry, these were measured, together with each flower segment over the UV-visible range between 300 and 700 nm using an Ocean Optics S2000+ spectrometer with a DT-mini light source and fiber optic probe (UV/VIS 400 μm). To assess the qualitative pattern of UV-absorbing and UV-reflecting parts of the flower, we photographed flowers using a UV camera (*Methods S1*).

Spectra was then imported into bee color space (Chittka et al. 1992) using hyperbolically transformed quantum catches and reflectance spectra of green foliage as the background. Furthermore, standard D65 daylight illumination was used. To determine whether (1) bees could perceive differences between the anther mimics and adjacent floral structures and (2) between the anthers and adaxial surface of the dorsal tepal, mean Euclidean distances with bootstrapped 95% confidence intervals were determined as chromatic contrasts. This was obtained using the *bootcoldist* function implemented in the R package “*pavo*” (Maia et al. 2019). Color distances below the perceptual threshold of 0.11 hexagon units is considered as indistinguishable by pollinators (Dyer 2006; Bukovac et al. 2017).

DO ANTHER MIMICS FACILITATE MORPHOLOGICAL FIT OF POLLINATORS WITH FLOWERS?

Physical structure of the anther mimic on pollen deposition

Single visits to virgin flower were used to test whether anther mimics enhance pollen deposition to receptive stigmas. We were able to directly link pollinator preferences for the physical structure of anther mimics (see the previous section) with pollen deposition to treatments in the following manner: after each foraging bout, the second observer identified the “preferred” flowers in the experimental array that received a single visit and carefully removed the stigmas of visited flowers near the base of the ovary. Stigmas were immediately placed in a labelled 2.5-mL centrifuge tube and maintained in an ice-filled cooler box while in the field. Experimental arrays were immediately reconstructed with fresh inflorescences maintaining a constant 10 inflorescence pairs all with virgin flowers. Once a new inflorescence was introduced, the positions of the treatment were swapped. Stigmas were embedded in heated fuchsin gel mounted on microscope slides later the same day. A dissecting microscope was used to count the total number of *T. laxifolia* pollen grains on each stigma. *Tritonia laxifolia* pollen was easily identified relative to other community members represented in a pollen library of the site.

To account for the high number of zeroes in the dataset, which led to overdispersion in an initial model using a Poisson

error distribution, we used a GLMM with a negative binomial error distribution and log link function (Zuur et al. 2009; Brooks et al. 2019). In our analysis, we removed butterflies from the full dataset, only retaining bees as the primary pollinators, although we report on both (i.e., butterflies act as nectar thieves within the Fish River Pass population). Treatments were as follows: flowers with their anther mimics excised and unmanipulated controls were assigned as fixed factors, and *T. laxifolia* pollen counts were treated as the response. Individual visitors were treated as random factors to account for nonindependence resulting from similar pollinator morphology and behaviors.

Physical structure of the anther mimic on pollen-collecting behavior

To assess whether pollen deposition from single visits is the consequence of pollen-collecting behavior exhibited by bees on anther mimics, we extracted behavioral data from 159 videos recorded during our field season at Mosslands. We set up arrays similar to the experiments investigating morphological fit described above. This yielded 43 videos of bees on flowers with the anther mimics excised and 116 on flowers as unmanipulated controls. These videos were recorded using a Canon 5D MKIV with a 100-mm USM macro lens shot at 30 fps. We recorded two kinds of behaviors exerted by bees: pollen-collecting behavior in the form of scraping or pulling on the anther mimics, and nectar foraging. Although demonstrating pollen-collecting behavior on excised anther mimics upon entrance of the flower can be interpreted as a behavior to simply enter the floral tube, we only recorded pollen-collecting behavior on excised anther mimics if bees momentarily scraped their front tarsi on the two-dimensional structure (Video S1).

GLMMs with a binomial error distribution and a logit link function were used to determine if statistical differences in pollen-collecting behaviors exists between treatments (anther mimics retained and excised). Two models were run, namely, the presence or absence of pollen-collecting behavior on anther mimics between treatments including the proportion of pollen-collecting behavior that resulted in contact with the reproductive parts between treatments. In both models, bee individual was included as a random factor to account for nonindependence regarding individual bee behavior.

DO PREFERENCES FOR AND MORPHOLOGICAL FIT ON ANTHR MIMICS HAVE CONSEQUENCES FOR SEED SET?

To determine whether anther mimics are associated with female reproductive success. We compared seed set from treatments

with their anther mimics excised from unmanipulated controls of naturally occurring rooted plants within the population. Similar to the previous two experiments, we emasculated all experimental flowers and excised anther mimics from a total of 46 flowers (anther mimics removed) and left anther mimics of 51 flowers intact (unmanipulated controls), providing a total of 97 experimental flowers. We covered 23 treatments with their anther mimics excised and 29 unmanipulated controls (52 treatments) with 33 chicken wire boxes rooted with steel tent pegs. We did this to determine if butterflies made a significant contribution to fitness. If this was the case, we expect open treatments to experience a higher proportion of seed set than caged individuals. Wire cages had holes large enough to allow bees (*A. fallax* body length: distance from head to tip of abdomen, 11.07 ± 0.97 mm [$n = 4$]) to enter the cages (25 mm holes) (see Fig. S2e), but small enough to prevent white butterflies from entering (Authors, pers. obs.). The remaining 32 inflorescences were left uncaged, containing 28 manipulated and 17 unmanipulated flowers (45 treatments). After 3 weeks, we collected fruits and discerned fertilized from aborted ovules. Fertilized ovules were much larger, hard, and green in appearance, whereas aborted ovules were smaller, soft, and shriveled in appearance (see Fig. 1d).

Of the 33 cages setup initially, five were destroyed by cattle, leaving a total of 28 cages intact. Fruit set from the five destroyed cages were discarded from the analysis. Statistical differences in seed set among treatments (anther mimics retained and excised) and exposure (caged vs. open) and their interaction were calculated using GLMM with a beta-binomial error distribution and a logit link function. Individual inflorescences were treated as a random factor to control for multiple treatments per inflorescence.

All GLMMs were calculated using the *glmmTMB* command from the package “*glmmTMB*,” significance of fixed effects was determined using the ANOVA, type III command from the “*car*” package (Bolker et al. 2009), and contrasts among interaction terms for the selection experiment were determined using the *emmeans* command from the package “*emmeans*.” All models were checked using the “*DHARMA*” package. In the process of checking the models, we discovered that the final model was overdispersed, and we corrected for overdispersion using a beta-binomial error distribution to model the proportion of seed set from the selection experiment (Harrison 2015). Median values and 90% confidence intervals for plotting were obtained from model predictions using 1000 bootstrap samples calculated using the *bootMer* command from the package “*lme4*.” All data analysis was conducted using the R statistical environment (R Development Core Team 2021).

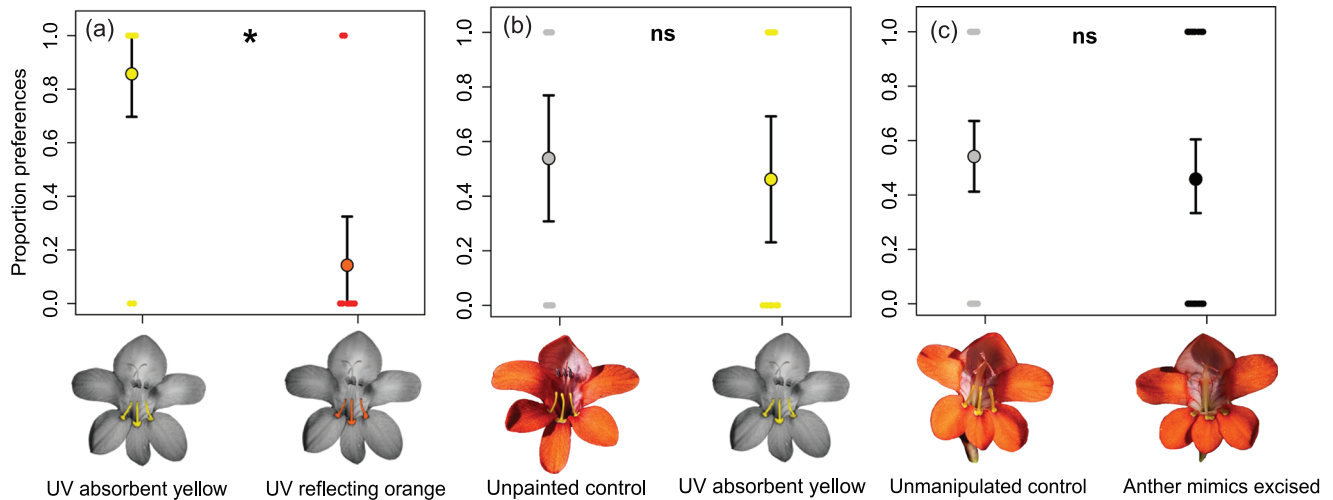


Figure 3. Binary preferences of bees based on their first choices (a) between anther mimics painted with yellow UV-absorbent paint and orange UV-reflecting paint; (b) choices between unpainted controls with naturally yellow anther mimics and anther mimics painted with yellow UV absorbent paint; and (c) choices between unmanipulated controls and flowers with anther mimics excised. Colored circles represent median model predictions, error bars refer to 90% confidence intervals for model predictions, and small colored points represent balanced binary preferences for each treatment. *ns* = $P > 0.05$, * $P < 0.05$.

Results

DO POLLINATORS PREFER THE COLOUR AND STRUCTURE OF ANTHER MIMICS?

Color preferences

At Mosslands, 14 *A. fallax* bees showed a significant selection bias for flowers with anther mimics painted with yellow UV-absorbent paint over flowers painted with orange UV-reflecting paint ($\chi^2 = 11.00$, $df = 1$, $P < 0.001$; Fig. 3a), the loci of UV-reflecting orange paint being close to the loci of the orange of adjacent flower tepals in bee color space (Figs. 2d, 4, S3, S4a). In contrast, 13 *A. fallax* bees made equal choices between anther mimics painted with yellow UV-absorbent paint over unpainted controls ($\chi^2 = 0.15$; $df = 1$, $P = 0.70$; Fig. 3b), the loci of both these yellow colors clustering together in bee color space (Figs. 4, S4b). Chromatic contrasts reveal that anther mimics were above the threshold of 0.11 hexagon units (Dyer 2006; Bukovac et al. 2017) when compared to all other floral traits (Figs. 4, S3, S4c). However, the anthers were perceptually similar to the adaxial surface of the dorsal tepal, being well below the threshold of 0.11 hexagon units (Fig. S4d).

Preference for the physical structure of the anther mimic

At Fish River Pass, we interviewed 40 insects, of which 20 were bees (16 *A. fallax* and four *A. mellifera scutellata*) and 20 were butterflies (19 *C. eris eris* and one *P. eriphia eriphia*), which made a total of 88 preferences. The model including all insects (both bees and butterflies) ($\chi^2 = 3.81$, $df = 1$, $P = 0.051$) showed

no significant preference for flowers with or without physical anther mimics present. Removing butterflies from the dataset did not alter this result and bees alone showed no preference for flowers with or without anther mimics ($\chi^2 = 0.66$, $df = 1$, $P = 0.415$; Fig. 3c).

DO ANTHER MIMICS FACILITATE MORPHOLOGICAL FIT OF POLLINATORS WITH FLOWERS?

Physical structure of the anther mimic on pollen deposition

At Fish River Pass, we obtained a total of 74 single visits from 34 insects. Of these, 41 visits were made by bees (14 *A. fallax* and two *A. mellifera scutellata*) and 33 by butterflies (17 *C. eris eris* and one *P. eriphia eriphia*). Pollinators deposited significantly more pollen grains onto virgin stigmas of unmanipulated controls, compared to flowers with their anther mimics removed ($\chi^2 = 6.70$, $df = 1$, $P = 0.009$). This result did not change when butterflies were removed from the dataset, which highlights the significant contribution of bees to pollen deposition in the experiment ($\chi^2 = 5.74$, $df = 1$, $P = 0.017$; Fig. 5).

Importance of anther mimic structure for pollen-collecting behavior

At Mosslands, we recorded 155 *Amegilla fallax*, a single *Alodape*, and a single Halictid bee visiting 42 flowers with the anther mimics excised and 113 unmanipulated control flowers. Here, bees demonstrated a higher proportion of pollen-collecting behavior on unmanipulated flowers with their anther mimic

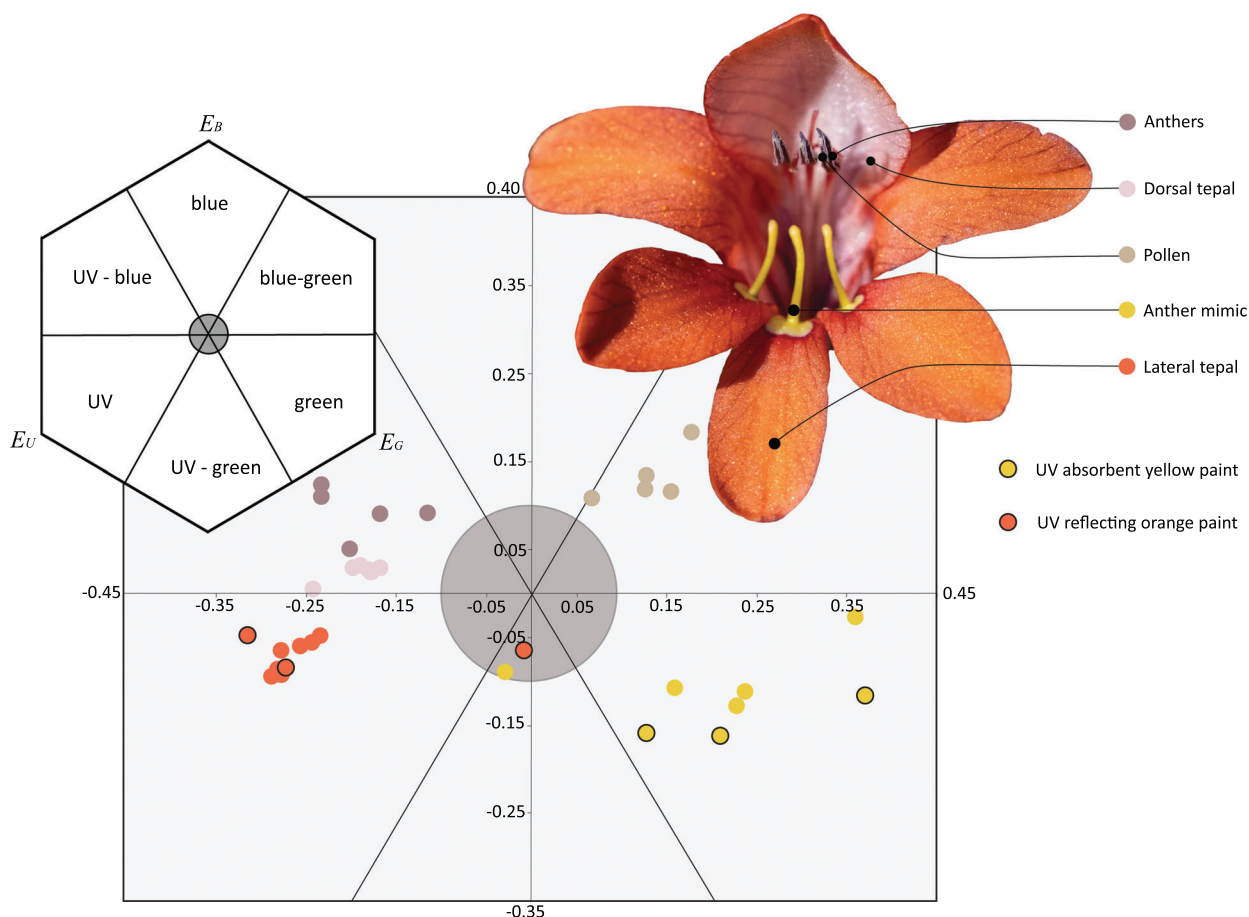


Figure 4. Color spectra from different parts of the flower plotted in bee color space (inset). Colors represent actual colors from respective parts of the flower as perceived by humans. Points with a black outline are measured from anther mimics painted with either orange UV-reflecting paint or yellow UV-absorbing paint. Spectra in the central gray circle appear achromatic to bees (0.1 hexagon units).

retained, compared to flowers with their anther mimics excised (57.14%) ($\chi^2 = 10.14$; $df = 1$, $P = 0.001$; Fig. 6).

Contact with reproductive parts and associated pollen-collecting behavior

The proportion of bees contacting anthers and stigmas was significantly higher on unmanipulated controls versus flowers with anther mimics excised ($\chi^2 = 30.87$, $df = 1$, $P < 0.001$). The result remained similar when nectar foraging bees were excluded and only pollen-collecting bees considered ($\chi^2 = 11.62$, $df = 1$, $P < 0.001$).

DO PREFERENCES FOR AND MORPHOLOGICAL FIT ON ANTHR MIMICS HAVE CONSEQUENCES FOR SEED SET?

Butterflies did not contribute significantly to seed set at the Fish River Pass site, as the proportion seed set of the plants caged to exclude butterflies were similar to that of the controls ($\chi^2 = 0.33$, $df = 1$, $P = 0.69$; Fig. 7). However, the removal of the physical anther mimics led to a significant decrease in the proportion seed

set ($\chi^2 = 10.90$, $df = 1$, $P < 0.001$; Fig. 7). There was no significant interaction between pollinator exclusion and anther mimic excision, and none of the contrasts between caged and open treatments were significant ($\chi^2 = 0.08$, $df = 1$, $P = 0.78$; Fig. 7).

Discussion

Our results demonstrate that *T. laxifolia* is pollinated by bees through nectar foraging and multimodal floral mimicry. Multimodal floral mimicry is achieved through both the color preferences of bees and pollen-collecting behaviors directed at the three-dimensional anther mimics. Furthermore, we demonstrate that the pollen reward is concealed against the dorsal tepal through crypsis, which has the potential to divert the attention of the bees to the UV absorbent color signal of the anther mimic. Besides the importance of both these visual and tactile signals, our data show that the three-dimensional anther mimics play a crucial role in precisely positioning pollinators to deposit pollen on stigmas and presumably remove pollen from anthers.

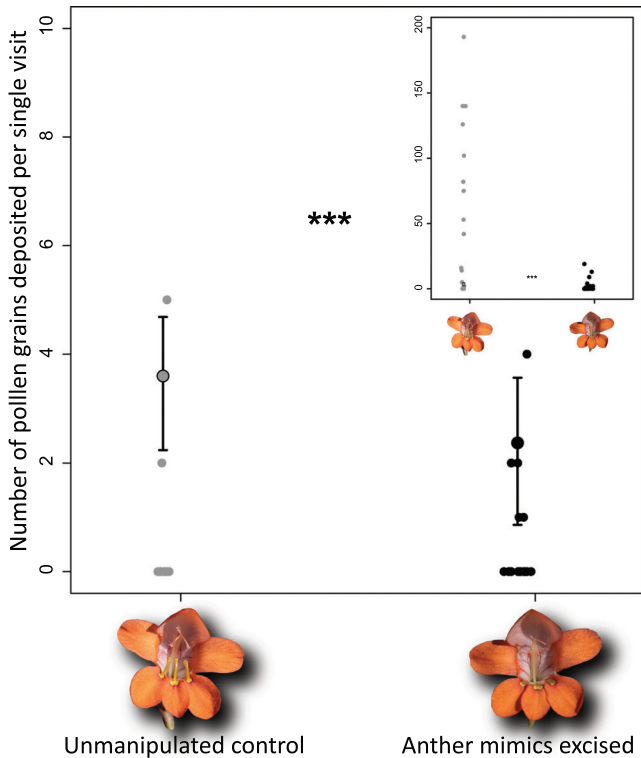


Figure 5. Unmanipulated flowers of *Tritonia laxifolia* with anther mimics present received significantly more pollen on their stigmas following a single visit by a pollinating bee compared to flowers with their anther mimics excised. Colored circles represent median model predictions, error bars refer to 90% confidence intervals for model predictions, and small points represent the number of pollen grains deposited for each single visit replicate. Inset shows full extent of data points. *** $P < 0.001$.

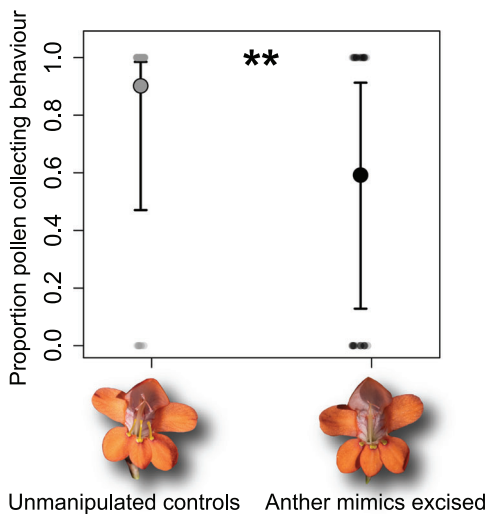


Figure 6. Proportion of pollen-collecting behavior on unmanipulated controls and flowers with excised anther mimics. Colored circles represent median model predictions, error bars refer to 90% confidence intervals for model predictions, and small points represent binary outcomes (i.e., presence or absence of behavior exhibited). ** $P < 0.01$; *** $P < 0.001$.

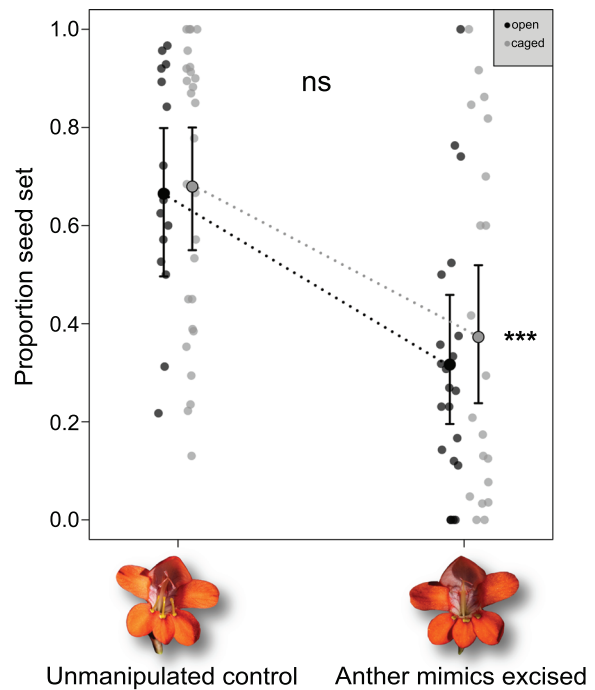


Figure 7. Mean proportion of seed set from caged and open treatments on rooted unmanipulated controls and flowers with their anther mimics excised. Manipulated caged and open treatments experience significantly lower seed set compared to unmanipulated caged and open treatments. However, there is no significant difference in seed set within manipulated and unmanipulated treatments for caged versus open treatments, suggesting that the presence of butterflies in open treatments did not contribute significantly to seed set. ** $P = 0.001$.

Yellow UV-absorbing floral signals are considered important in orientating pollen-foraging insects to flowers (Lunau 2014), and laboratory experiments using untrained naïve bumblebees (*Bombus terrestris*) demonstrate preferences for the visual signals of anther mimics by orientating themselves toward the pollen signal of dummy pollen and touching the mimics with their antennae (see Lunau [2000] and Wilmsen et al. [2017]). Bees in our study selected flowers with anther mimics painted with yellow UV-absorbent paint exclusively over flowers with mimics painted with orange paint that reflected UV. The paints used for these manipulations approximate the respective floral parts in bee color space, and the bees are unlikely to be able to distinguish the UV-absorbent yellow paint from the unpainted yellow anther mimics, or the orange UV-reflecting paint from the tepals in preference experiments. This is supported by mean Euclidean distances with confidence intervals that are either less than or overlaps with the perceptual threshold of 0.11 hexagon units (Dyer 2006; Bukovac et al. 2017) (Figs. 4, S4a). Similar experiments altering the UV color signal by applying sunscreen to flowers resulted in reduced preferences by bees (Johnson and Andersson 2002), which may have consequences for

reproductive success. An underlying explanation for the influence of color alteration on bee preferences may be associated with the spectral purity of the painted anther mimics as demonstrated in experiments looking at preferences of naïve bees on dummy pollen (Lunau et al. 1996, 2006). Nevertheless, alteration of the UV signal in our study may have led to a higher proportion of visits to flowers with the UV-absorbent color signal, similar to that of pollen, which may have been the result of bees specifically foraging for pollen rewards as indicated by a higher proportion of pollen-collecting behaviors on unmanipulated controls when compared to nectar foraging (see Fig. S5).

In bee color space, the color of the pollen and anthers contrasted strongly with that of all other floral parts except the pink dorsal tepal directly behind the anthers (Fig. S4). We interpret this as a case of crypsis to prevent pollen-collecting insects from discovering the anther and reducing male fitness by collecting pollen as a reward (see Xiong et al. 2019). By camouflaging the pollen against the background of the dorsal tepal, *T. laxifolia* deceptively directs attention to the yellow UV-absorbing signal of the anther mimics. However, we have observed that bees do collect pollen from flowers by pushing their head against the anthers and grooming themselves directly afterward, often occurring following an attempt to remove pollen from the anther mimics (Video S1). Together with the nectar reward, this behavior may act as a trade-off to ensure that bees return to flowers, and it is likely that this occurs later in the season by more experienced bees. However, more research is required to confirm this notion.

In contrast to our findings, Duffy and Johnson (2015) showed that yellow anther mimics and pollen are virtually indistinguishable to bees. In their system, this convergence of color may have evolved to increase the display of the pollen reward and increase visitation time on flowers that may improve reproductive success. This idea is supported in the removal of anther mimics resulting in decreased preferences to flowers with excised anther mimics and consequently seed set. When we provided pollinators choices between flowers with the anther mimics removed and unmanipulated flowers, pollinators did not show any preference. This was because the excision of the pollen mimic did not remove the yellow UV-absorbing pollen signal but made it two-dimensional instead. Therefore, we found no significant preference for the three-dimensional structure of the pollen mimic, which contradicts our expectation for increased preferences for larger UV-absorbent signals (see Fig. 1). Similar experiments have been conducted on *Jacaranda rugosa* (Bignoniaceae) by Milet-Pinheiro and Schlindwein (2009) that show decreased visitation to flowers with their staminodes excised. However, in their study, the excision of the staminode removes the yellow UV-absorbent signal that is comparable to our first experiment where we painted the anther mimics the same color as the tepals that resulted in no visits by pollinators.

Despite the lack of choices made to the three-dimensional structure of the anther mimic, pollinators transferred significantly more pollen per single visit on the stigmas of unmanipulated controls versus flowers with anther mimics excised. This is, in part, the result of the three-dimensional structure of the anther mimic that decreases the width of the flower entrance between the anther mimic and the reproductive part of the flower. Preliminary observations by the specialist on the genus state that “The function of the calli (anther mimics) is probably to diminish the space in the throat of the perianth, thus ensuring that a visiting insect will brush with its back against the anthers and stigmas” (de Vos 1982). Indeed, the distance between the closest stigma branch and the top of the anther mimic on the median tepal is 3.45 ± 0.19 mm ($n = 20$), which is 1.28 mm less than the thorax height of the most abundant bee pollinator *A. fallax* 4.73 ± 0.07 mm ($n = 12$) (Methods S2). Importantly, less abundant butterflies fit poorly with flowers and the anther mimics. From observations and photographic evidence (Fig. S2B), the relatively long proboscides of the butterflies visiting the flowers result in the insects probing the flowers between the anther mimics with their heads remaining outside of the flower. As a consequence, butterflies had remarkably low pollen loads, and we did not find a single pollen grain from *T. laxifolia* on any of the wings or heads of the 12 butterflies we swabbed for pollen loads. In contrast, *A. fallax* carried 128.9 ± 40.7 SE ($n = 10$) pollen grains on dorsal section of their thorax (Methods S3).

The large pollen loads borne by *A. fallax* translated into the substantial number of pollen grains deposited on virgin stigmas in single visit experiments. Virgin stigmas of unmanipulated controls with intact anther mimics received the highest pollen loads compared to control flowers with their anther mimics removed. Few studies have looked at the effects of structural three-dimensional anther mimics in enhancing the morphological fit between flowers and pollinators. The most rigorous studies that do test this have focused mainly on taxa with vestigial staminodes. For example, Dieringer and Cabrera (2002) found a statistical difference for pollen deposition in *Penstemon digitalis* when comparing control flowers with their staminodes intact, with flowers with their staminodes excised. Similar findings were made by Walker-Larsen and Harder (2001a) for bee-pollinated *Penstemon ellipticus* and *Penstemon palmeri*, but not for hummingbird-pollinated *Penstemon centranthifolius* and *Penstemon rostriflorus* with their staminodes retained and excised. None of these studies associate pollen-collecting behavior with reproductive success, although there is evidence that the presence of staminodes increases the time spent by pollinators within the flower.

Our videos of the behaviors of bees visiting experimental and control flowers allow us to make a direct link between pollen-collecting behavior and the amount of contact made with the

reproductive organs. Specifically, we found that pollinators displayed a higher proportion of scraping and pulling behavior on flowers with intact anther mimics (Fig. S5; Video S1). This has fitness implications for two-dimensional pollen-imitating markings versus three-dimensional anther mimics with regard to attraction and morphological fit with flowers. Based on our results, pollen-imitating markings seem to play an important role in attraction (Fig. 3a), whereas three-dimensional anther mimics may be important in both attraction and in eliciting pollen-collecting behavior. Importantly, Wilmsen et al. (2017) showed that multimodal stimuli (e.g., visual and tactile) are required to elicit full behavioral responses by bees, which was similar to the responses exhibited on actual Dandelion pollen. In a sexual deception system, Ellis and Johnson (2010) showed that ray-florets of the daisy *Gorteria diffusa* with three-dimensional floral signals elicited more mating attempts by male flies compared to plants with two-dimensional floral signals, resulting in more pollen export by the three-dimensional deceptive forms.

In our study, behavior on three-dimensional anther mimics is associated with a higher proportion of contacts to the reproductive parts of the flower, which is directly linked to pollen deposition and potentially seed set in selection experiments. To our knowledge, this is the first evidence for pollen-collecting behavior on three-dimensional anther mimics that improves the morphological fit between flower and pollinators (see Lunau et al. 2017). Ideally, documenting behavior on treatments with the three-dimensional structure kept intact but painted with the same color as the tepal to remove the color signal would be informative regarding the importance of the physical structure eliciting a tactile response in combination with the color signal (visual response). However, this proved difficult to execute due to few visits by bees to such treatments in our preferences. Nonetheless, pollen-collecting behavior alone does not result in pollination; nectar foraging also forces the pollinator to clamber over the anther mimics to access the reward, leading to a higher proportion of contacts to the reproductive organs in unmanipulated controls (Fig. 6). However, pollen-collecting behaviors on anther mimics seems to dominate in the population, as unmanipulated flowers received twofold more pollen-collecting behaviors compared to nectar foraging per visit, as recorded on video (Fig. S5; Video S1). This is likely the result of bees collecting pollen from several potential model flowers containing pollen with the same yellow UV-absorbent signal at our study localities (see hindlegs of bees in Fig. 1c). These include *Pauridia* sp. (Amaryllidaceae) and *Gazania* sp. (Asteraceae) that co-occurs with *T. laxifolia*.

In conclusion, our study makes the link between female reproductive success and the processes underlying the evolution of anther mimicry in *T. laxifolia*. We show that the yellow UV-absorbent pollen signal is important in the visual attraction of pollinators to flowers and that the three-dimensional structures not

only elicit pollen-collecting behaviors, but such behaviors lead to improved pollen deposition and consequently seed set. Future studies should focus on the generality of pollen-collecting behaviors on pollen-imitating structures, and whether inexperienced naïve bees exhibit a higher proportion of pollen-collecting behaviors on these structures compared to more experienced bees.

AUTHOR CONTRIBUTIONS

Data collection was performed by all authors. Data analysis was performed by KK, EN, and CP. EN wrote the first draft. All authors contributed to the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA ARCHIVING

Data are available at Dryad: <https://doi.org/10.5061/dryad.f1vhhmh02>.

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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 A subset of species from the genus *Tritonia* (Iridaceae) with structural variation in their anther mimics.

Fig. S2 A) Study site where we performed preference and selection experiments near Fish River Pass, Pikoli in the Eastern Cape Province of South Africa.

B) Gold tip butterfly *Colotis eris eris* thieving nectar from flowers of *Tritonia laxifolia* at the study site. Notice the lack of contact made to the anthers.

Fig. S3 Images on the left represent unpainted and unmanipulated flowers, images on the right have UV reflecting orange paint added to the anther mimics.

Fig. S4 Chromatic contrasts based on Euclidean distances between spectra of different floral traits plotted in bee colour space.

Fig. S5 Spine plot illustrating the proportion pollinator behaviour exhibited per single visit by bees.

Video/Movie S1 Video clip illustrating pollen-collecting behaviour exerted on anther mimics by bees
Supplementary material