Original Article

Floral community predicts pollinators' color preference: implications for Batesian floral mimicry

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Animals that rely on nectar are expected to display floral trait preferences correlating to the signals of nectar source flowers. Batesian mimicry evolves to exploit these pre-existing signal–receiver relationships, attracting pollinators through an adaptive resemblance to specific co-occurring rewarding species. The nectar-feeding long-proboscid flies of South Africa are pollinators for several deceptive orchid species that are putatively Batesian mimics. We tested whether flies' measured color preference varied among communities providing different nectar-source diets, which would indicate the necessary signal–receiver conditions for the evolution of advergent Batesian mimicry. We introduced artificial rewardless flowers into flowering communities that supported divergent nectar diets in resident flies and inferred floral trait preferences of a long-proboscid fly species (*Prosoeca ganglbaueri*) from visitation behavior to these artificial flowers. The experiment showed that the preference of flies for white versus pink was strongly predicted by the colors of flowers most commonly visited by flies at a site. Furthermore, generalization in preference was positively correlated with the variance in nectar-community hue, i.e., flies showed more generalized preference in more spectrally diverse flower communities. The floral tube length of local nectar sources also influenced how readily the flies probed the artificial flowers during attempted foraging. These results support the hypothesis that nectarless orchids pollinated by *P. ganglbaueri* experience selection for traits that exploit site-specific mutualistic relationships between fly pollinators and their local floral communities.

Key words: **Batesian mimicry, color vision, conditioning, flower color, learning, pollination, vision**

INTRODUCTION

Mimicry presents many of the most compelling examples of adaptive evolution via natural selection. In floral mimicry, rewardless flowers attract their pollinators through dishonest signaling of a resource. The most common of these floral strategies is food-deception, where rewardless flowers mimic the morphology, color, and/or scent of rewarding flowers (Nilsson 1992; Jersáková et al. 2006).

Signaling in food-deceptive flowers ranges through a continuum of specialization (Jersáková et al. 2009; Schaefer and Ruxton 2009). The most generalized food-deceptive flowers present signals typically associated with floral rewards, but do not closely match a specific nectar-bearing species or pollination guild. Phenotypic variation is often high in generalized food-deceptive species and

they can attract a wide diversity of pollinating insects (Gigord et al. 2001; Jersáková et al. 2009; Johnson and Schiestl 2016). On the opposite side of the continuum are the most specialized food-deceptive flowers: Batesian floral mimics (Johnson and Schiestl 2016). The classic example of Batesian mimicry is where edible species of butterflies (mimics) avoid predation by mimicking a distasteful co-occurring species of butterfly (models) (Brower and Brower 1972). In flowers, Batesian mimics have no nectar but still attract pollinators by resembling more common co-occurring flowers that do have nectar rewards (Johnson 2000; Johnson and Schiestl 2016).

Batesian mimicry is not the only mechanism whereby similar floral phenotypes evolve in phylogenetically independent plant lineages. The most common path by which this occurs is via convergent evolution, when plants in different lineages are pollinated by animals in the same functional group (Fenster et al. 2004). In cases of convergence, pollinators from the same functional group share similar floral preferences, thereby exerting similar selective pressure

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on plant traits. Strong evidence for this is apparent in the global similarity of signals among different plants pollinated by particular animal groups (Rosas-Guerrero et al. 2014). In contrast, under true Batesian mimicry, similar floral phenotypes arise through advergent evolution, where the traits of the mimic are under selection to track the traits of model species, which must be sympatric (Brower and Brower 1972; Johnson et al. 2003). The criteria used to distinguish convergent evolution from true Batesian mimicry via advergent evolution are: 1) true Batesian mimics share their model species' habitat, phenology, and pollinators, 2) pollinators should have difficulty distinguishing between the mimic and model, 3) the pollinators' response to the mimic should be a misclassification based on learned response to the model, 4) the mimic should have higher fitness when it occurs with the model, and 5) mimics should occur at lower frequency than the model (Johnson et al. 2003; Anderson et al. 2005; Johnson and Schiestl 2016). While Batesian mimicry is rare, these conditions for mimicry have nonetheless been demonstrated in whole or in part for several systems (Nilsson 1983; Johnson 1994, 2000; Johnson et al. 2003; Anderson et al. 2005; Anderson and Johnson 2006; Newman et al. 2012; de Jager et al. 2016).

Of these criteria, item 3 is perhaps the most difficult to test as it requires demonstration that pollinators' attraction to the mimic is driven by prior experience with the model. This criterion draws a distinction between learned responses to signals, versus exploitation of sensory bias—a route to phenotypic similarity via convergence (Schaefer and Ruxton 2009). An important predicted outcome for this criterion is that, in true Batesian mimicry scenarios, the signal–receiver's behavioral response to the model should vary with prior experience. This can be tested in a plant–pollinator mimicry system by exploring how pollinator responses vary with changes in the background floral community, specifically where signal–reward associations differ among sites. Examining putative floral mimicry in a community context can thus reveal important information about whether it is generalized or Batesian. This is because while Batesian mimic fitness is correlated to resemblance with co-occurring model flowers, generalized mimicry should not be similarly dependent on floral community context (de Jager et al. 2016).

One of the clearest examples of the importance of background floral community for mimicry is seen in the butterfly-pollinated food-deceptive orchid, *Disa ferruginea*. In the west of *D. ferruginea's* range, its flowers are red and it co-occurs with the red-flowered model species *Tritoniopsis tritecea*, while in the east, *D. ferruginea* flowers are orange, and it co-occurs with the orange model *Kniphofia uvaria*. Both of the orchid ecotypes are preferred in their home range by local pollinators, which provides evidence of adaptive color shift and Batesian mimicry (Johnson 1994; Newman et al. 2012). In another study, *Orchis* orchids, which display a more generalized food deception, were more likely to share pollinators with rewarding flowers that had similar colors in a model of bee color vision (Gumbert and Kunze 2001). Outside these examples of the importance of background floral community driving advergent evolution of floral color in deceptive flowers, the interaction between community color variance and pollinator preference has rarely been studied. Our study therefore asks how color in a community of floral nectar sources influences preference in a keystone South African pollinator species known to visit species of putatively Batesian orchids.

South Africa is a hotspot for Batesian food-source mimicry, perhaps because of the high floristic diversity and frequency of specialized plant–pollinator interactions (Johnson and Steiner 2003;

Ollerton et al. 2006). A prime example of this floral specialization involves the guilds of plants pollinated by various specialist nectar-feeding long-proboscid tabanid and nemestrinid flies (Goldblatt and Manning 2000). The flowers of these plant guilds share common features such as long floral tubes, and a color range mostly restricted to white, cream and pink (as perceived by humans; Figure 1). While most of the guild members provide nectar rewards to their fly pollinators, there are also a surprisingly high number of rewardless orchid species, representing several repeated independent evolutions of putative floral Batesian mimicry (Johnson et al. 2013). Previous studies suggest that exploitation of mutualisms between long-proboscid flies and rewarding flowers requires that Batesian floral mimics match both the color and shape of the rewarding flowers (Johnson et al. 2003; Jersakova et al. 2012). To explore the interaction of fitness in floral mimics with flower color and flower shape in plant communities, we studied the long-proboscid fly *Prosoeca ganglbaueri* (Nemestrinidae), pollinator for a guild of approximately 30 plant species with long corolla tubes. This fly pollinates several deceptive orchids, including *Disa nivea*, *Disa amoena*, and *Disa oreophila* subsp. *erecta* (Johnson and Steiner 1995; Anderson et al. 2005; Anderson and Johnson 2009). Supporting the case for Batesian mimicry, close correlation in spectral reflectance and floral morphology has previously been demonstrated between *D. nivea* and co-occuring nectar source *Zaluzianskya microsiphon* (Anderson et al. 2005). While visitation and pollination by *P. ganglbaueri* is confirmed for *D. amoena* and *D. oreophila* subsp. *erecta* (Johnson and Steiner 1995; Goldblatt and Manning 2000), trait matching to potential model species has not yet been tested. As well as long-tubed plants adapted for visits by *P. ganglbaueri*, the fly also obtains nectar from a number of short-tubed plants that are adapted for pollination by other vectors.

This experimental study addresses a critical criterion of the mimicry hypothesis for the evolution of deceptive flowers pollinated by *P. ganglbaueri*, that is, visits to a Batesian mimic are a misclassification of learned preference. Via field experiments with artificial deceptive flowers varying in color, we test how the behavioral response of flies to deceptive mimics is influenced by the community of floral phenotypes they use for nectar. By using artificial flowers previously shown to elicit foraging behavior in specialist nectar-feeding flies (Jersáková et al. 2012), we simulate (under field conditions) how the color phenotypes of a Batesian mimic might interact with the traits of the background nectar-flower community to influence visitation, and therefore fitness.

METHODS

Study species and sites

We studied color preference in 7 populations of *P. ganglbaueri* spread over a range of 250 km in the Drakensberg Mountains, South Africa, during the summer flowering season of 2014. The 7 sites we used varied in their floral community composition, meaning that the nectar resources used by *P. ganglbaueri* varied among sites. At some sites, flies accessed nectar from only a single species of flower (Golden Gate, Sehlabathebe, Elands Height, Qacha's Nek; Table 1). At the other sites, the diversity of nectar sources was higher, with the fly accessing nectar from 3 or 4 species of plant. Quantification of fly visits to different plant species was made during the course of the behavioral experiment, described below. Deceptive orchids visited by *P. ganglbaueri* are rare, and we recorded the presence of nectarless orchids at only 2 of our sites: pink-flowered *Disa oreophila* subsp. *erecta* at Naude's Nek (known to be visited by *P. ganglbaueri*

Figure 1
Prosoeca ganglbaueri feeding from (a) *Zaluzianskya microsiphon*, (b) *Scabiosa columbaria*, (c) *Agapanthus campanulatus*, and (d) *Dianthus basuticus*.

Table 1

Study sites, location, and hue variance in the community of available floral nectar resources, measured in LM-MS color space via segment classification, and the color-opponent coding vision model of fly vision

Site	Code	Lat	Long	Var*	Var†
Sehlabathebe	SH	−29.877	29.072	0	0
Qacha's Nek	QN	−30.136	28.676	0	0
The Sentinel	SN	−28.727	28.891	0.0059	0.0070
Naude's Nek	NN	−30.733	28.140	0.0495	0.0415
Matatiele Dam	MD	−30.400	28.820	0.0293	0.0048
Golden Gate	GG	−28.506	28.619	0	0
Elands Height	EH	−30.794	28.227	0	0

Hue variance measured as angular variance of hues calculated in LM-MS color space via segment classification (*), and the color-opponent coding vision model of fly vision (†).

(Johnson and Steiner 1995)), and pink- and white-flowered forms of *Disa cephalotes* at Sentinel (pollinated by smaller species of *Proseoca* and *Philoliche* flies (Johnson et al. 2003)). We therefore sampled spectral reflectance data from 2 putative Batesian mimic orchid species from additional sites: white-flowered *Disa nivea* from Sani Pass, KwaZulu-Natal, and pink-flowered *Disa amoena* from Long Tom Pass, Mpumalanga.

Behavioral experiment

To quantify fly color preference, we constructed acetate "interview bouquets" following the protocol of Jersáková et al. (2012).

In fly pollinators, these artificial flowers elicit attraction and probing responses that are statistically indistinguishable from those elicited by real flowers (Jersáková et al. 2012). Transparent acetate was painted with acrylic paint, then cut and rolled into floral tubes with dissected edges simulating the appearance of petals. The artificial flowers were painted to match either the white or pink reflectance spectra of *Prosoeca*-pollinated flowers (Figure 3, Supplementary Figure S1). Seven flowers per bouquet were attached to a bamboo kitchen-skewer in a raceme arrangement. The interview bouquets were then used in a paired choice experiment using the presentation stick method developed by Thomson (1981). Interview bouquets were attached to opposite ends of the cross-beam on a "T" bar (40 cm bar mounted perpendicular to the end of a 1.5 m pole). This could then be lowered to approximately 30 cm from an actively foraging fly. We attempted a choice trial on every occasion we observed a fly foraging, or perched in the field. For each trial we used a voice recorder to note 1) the plant species most recently visited by the individual, 2) the color of the interview bouquet chosen by the fly, and 3) the plant species the individual visited immediately after each choice on the interview bouquet. We considered a choice made when the foraging fly made a close inspection flight or probed a flower on a bouquet. We scored the trial as a "reject" where no choice was made.

Flower color spectra and fly vision modeling

We quantified the colors of natural nectar-bearing flowers visited by *P. ganglbaueri* at each site, as well as 3 deceptive orchids known to be visited by *P. ganglbaueri*. To do this, we collected 2–5 flowers from different individual plants, and measured their spectral reflectance

over the UV-visible range (300–700 nm) using an Ocean Optics S2000 spectrometer with an Ocean Optics DT-mini light source (200–1100 nm; Dunedin, FL, USA) and fibre optic reflection probe (UV/VIS 400 μm) held at 45°. At least 2 measurements were taken per specimen and spectra were averaged for analyses.

We modeled fly perception of flower colors and artificial flowers using a color-opponent coding (COC) vision model (Troje 1993) and the generic receptor noise limited (RNL) color-opponency model (Vorobyev and Osorio 1998). The COC model uses the relative quantum catch of flies' 4 photoreceptor types to plot spectra as loci in 4 quadrants of a Cartesian plane, where loci within a quadrant represent colors that are too similar to be distinguished, but loci in different quadrants are likely to have perceptible color differences. We applied this model following Ohashi et al. (2015) and Jersáková et al. (2012) with photoreceptor quantum catches hyperbolically transformed (Hempel de Ibarra et al., 2014), under D_{65} standard daylight illuminant, and ran analyses for standard leaf green background (Dafni et al. 2005) and an average green background from grassland habitat representative of our study sites (Shuttleworth and Johnson 2009). We modeled spectral reflectance data separately under both of the 2 available fly receptor sensitivity curves for *Lucilia* (Troje 1993), and *Eristalis* (Lunau 2014; Shrestha et al. 2016).

Vorobyev and Osorio's (1998) receptor noise limited (RNL) model also uses spectral sensitivities and the relative abundances of the photoreceptor types, but provides a measure of the perceived color contrast between objects (ΔS, as Just Noticeable Differences [JND]). If JND > 1, color discrimination is likely. The relative proportions of each photoreceptor (1:2:1:2) are from Earl and Britt (2006) and a Weber fraction of 0.1 was used (Vorobyev and Osorio 1998). Quantum catches were converted into co-ordinates in tetrahedral space using equations A8–A12 from Kelber et al. (2003). We used spectral sensitivity datasets for 2 species of flies, both of which are tetrachromats with very similar regions of peak receptivity in the UV, blue, yellow, and purple regions of the spectrum (Supplementary Table S2).

Despite the wide phylogenetic and ecological diversity of flies, there are only 2 color vision models available for flies: a categorical model specifically developed for flies (Troje 1993) and a generic tetrachromat model (Vorobyev and Osorio 1998). These models can give conflicting results (Kelly and Gaskett 2014; Bodley et al. 2016) and comparative validation of the 2 models with behavioral experiments are lacking. The Troje (1993) COC model is supported by behavioral data from lab populations of *Lucilia cuprina* blowflies (Calliphoridae) (Fukushi 1994) and has been applied in studies to represent color vision of flies from the families Empididae, Mycetophilidae, Syrphidae, and Tabanidae (Jersáková et al. 2012; Kelly and Gaskett 2014; Ohashi et al. 2015; Bodley et al. 2016; Shrestha et al. 2016). Vorobyev and Osorio's (1998) receptor noise limited model can be widely applied to a range of tetrachromats (Kelber et al. 2003; Brembs and de Ibarra 2006; Kelber and Osorio 2010). It is well validated for taxa such as birds, but has been validated with behavioral tests for only one fly: lab populations of *Drosphila melanogaster* (Brembs and de Ibarra 2006). As far as we are aware, no studies test both of these models simultaneously in behavioral experiments of natural fly populations.

Statistical analysis

To test for pink or white flower preference in flies, we analyzed the interview data with binomial generalized estimating equations (GEE) in SPSS v. 21 (IBM Corp). We assigned each observed foraging bout as the subject to account for potential nonindependence resulting from repeated measures of the same fly individual. We used an exchangeable correlation matrix, logit link, with site as factor, foraging bout as the repeated subject variable, and the dependent variable being a binary choice (either approach or probe) for pink (1) or white (0). Significance of a preference was determined by plotting estimated marginal mean choice and 95% confidence intervals relative to a no preference mean of 0.5. To further explore factors that influence probing behavior in fly foraging, we used logistic regression to analyze the relationship between the proportion of nectar resources being used that had long corolla tubes and the proportion of approaches to the interview bouquet that resulted in probing behavior. We also analyzed data from 2 sites (Sentinel and Naude's Nek) at which flies were using both short and long-tube flowers for nectar resources to test whether flies' behavioral responses to the interview bouquets differed according to the morphology of the flower being visited at the beginning of each trial. To do this, we carried out binomial GLMs to test the influence of nectar flower tube length (defined as short-tubed = < 10 mm, long-tubed = > 15 mm) on both the probability of probing the interview bouquets, and the proportion of choices for pink over white. Post-hoc comparisons among means were done with the Dunn-Sidak method. Independence among sites was confirmed by carrying out a Mantel test (10,000 Monte-Carlo replicates) of similarity in the distance matrices for geography and average site residual from the binomial GLM in R (R Core Team 2013). Euclidean distance matrices were calculated from GPS coordinates converted to UTM.

To calculate an objective measure of the color variability in the natural nectar-bearing flower communities, we used the site-averaged spectra for each plant species on which we observed *P. ganglbaueri* feeding. These site-averaged spectra were then plotted as loci in 2 different 2-dimensional visual models; Troje's COC model with *Lucilia* spectral sensitivities (Troje 1993) and LM-MS color space via segment classification analysis implemented in the R package *pavo* (Maia et al. 2013). Each locus was then converted to an angle representing hue. A community hue variance was then calculated for each community as the circular variance (to measure dispersion of angles) among all species' average hues (calculated in R package *CircStats*) weighted by the frequency of total pollinator visits to each species observed during preference trials. In this way, our measure of the variance of nectar-flower color is a functional variance based on resource usage by *P. ganglbaueri*, rather than the strict relative representation of each plant species in the community. Lastly, we calculated the strength of flies' color preference as the absolute value of the magnitude difference between mean binary preference at a site and no preference (0.5). To test for a bivariate correlation between community hue variance and the strength of preference at each site, we calculated Pearson's r in SPSS v. 21 (IBM Corp).

To provide statistical comparison of the loci of the flowers in the tetrahedral color space generated by Vorobyev and Osorio's color opponency model, we ran a Principal Components Analysis of the loci (as generated from the quantum catches, described above). This generated 3 Principal Components, that described 62.6%, 35.4%, and 2.0% of the variation in the distribution of the loci in the tetrahedral space. We used analysis of variance with Tukey post-hoc tests on the Principal Component scores to test whether natural flower species and the interview bouquets clustered significantly differently from each other (and was therefore in a significantly different location in the color space).

RESULTS

Our visitation data represent every observed foraging bout during the study. Across the 7 sites we observed *P. ganglbaueri* visiting flowers 465 times, encompassing a total of 17 plant species (Supplementary Table S1), with 10 of these species receiving more than 2 visits. With the exception of the blue flowers of *Agapanthus campanulatus*, most observed visits were made to flowers that fell in the white to pink, and pink to violet color ranges, as perceived by humans. Sites varied widely in the composition of nectar sources visited by *P. ganglbaueri* (Figure 2). At some sites, *P. ganglbaueri* was observed visiting only white flowers (Sehlabathebe and Qacha's Nek), or only pink flowers (Golden Gate and Eland's Height). A more generalist pattern of visitation was observed at other sites where several plant species of differing floral color provided the nectar diet of *P. ganglbaueri* (Sentinel, Naude's Nek, and Matatiele Dam).

Floral spectral reflectance

Overall, the artificial white flower showed a similar reflectance curve to *Scabiosa columbaria* and *Cephalaria sp.*, and differed slightly in peak reflectance, which was at shorter wavelengths (Supplementary Figure S1). The artificial white flower differed from flowers of *Zaluzianskya microsiphon* most notably in the reflection of blue wavelengths (400–500 nm), which were higher in the artificial flower. Comparing among white flowers; *Z. microsiphon* reflected some UV, while *Cephalaria* and *S. columbaria* did not. Among pink flowers, main peak reflectance was in the red and indigo regions of the spectrum, and some species reflected UV (e.g., *Brunsvigia grandiflora* and *Gladiolus oppositiflorus*). The pink artificial flower and the pink natural flowers had very similar reflectance across the spectrum, however artificial flowers had a lower overall reflectance. Blue flowers in the study showed peak reflectance in the blue and red regions of the spectrum.

Reflectance spectra of the 3 nectarless orchids resemble those of some of the rewarding flowers used by *P. ganglbaueri* (Supplementary Figure S1). The off-white *D. nivea* closely matched the patterns of reflectance peaks seen in the averages for *Z. microsiphon* populations and showed the same peak as the white artificial bouquet at around 400 nm. Pink *D. amoena* showed similar reflectance to the pink artificial bouquet but had a lower red peak, and while peaks in its spectra were similar to *Brunsvigia grandiflora* and *Gladiolus oppositiflorus*. Pink *D. oreophila* subsp. *erecta* showed similar reflectance to the pink artificial bouquet, with a higher red peak, and most closely resembled the *Dianthus* spectra.

Interview bouquet experiment

We conducted a total of 323 individual pink versus white choice trials with the artificial flowers. Of these, 31.6% resulted in the test fly probing at least one artificial flower, 52.9% resulted in an unambiguous approach to one artificial inflorescence, and in 15.5% of trials the artificial flowers were rejected or ignored. Preference for pink or white departed significantly from 0.5 at 6 out of 7 sites (Figure 3), and in each of these cases, the direction of the preference corresponded with the color of the flower most commonly used by flies. Flies at Qacha's Nek, Sehlabathebe, and Sentinel displayed white preference while those at Matatiele Dam, Golden Gate, and Eland's Height showed pink preference. There was no correlation between color preference and geographical distance matrices ($P = 0.6126$). Community hue variance in LM-MS color space ranged between 0 at sites of single flower colors, to 0.0495 at the most diverse site: Naude's Nek (Table 1). When calculated in the COC fly-vision model, hue variance varied from 0 to 0.0415. A negative correlation was found between variance in hue of nectar-resource plants (measured 2 ways) at a site and the overall strength of pink–white preference (LM-MS: Spearman $r = -0.906$, $P = 0.005$; COC: Spearman $r = -0.867$, $P = 0.012$) (Figure 4).

Among floral communities, we found a positive relationship between the probability that flies probed the artificial flowers and the proportion of nectar plants being used that have long corolla tubes (Figure 5), likelihood-ratio $\chi^2 = 15.837$, $P = 0.000$. In communities where flies fed on both long- and short-tubed flowers, they
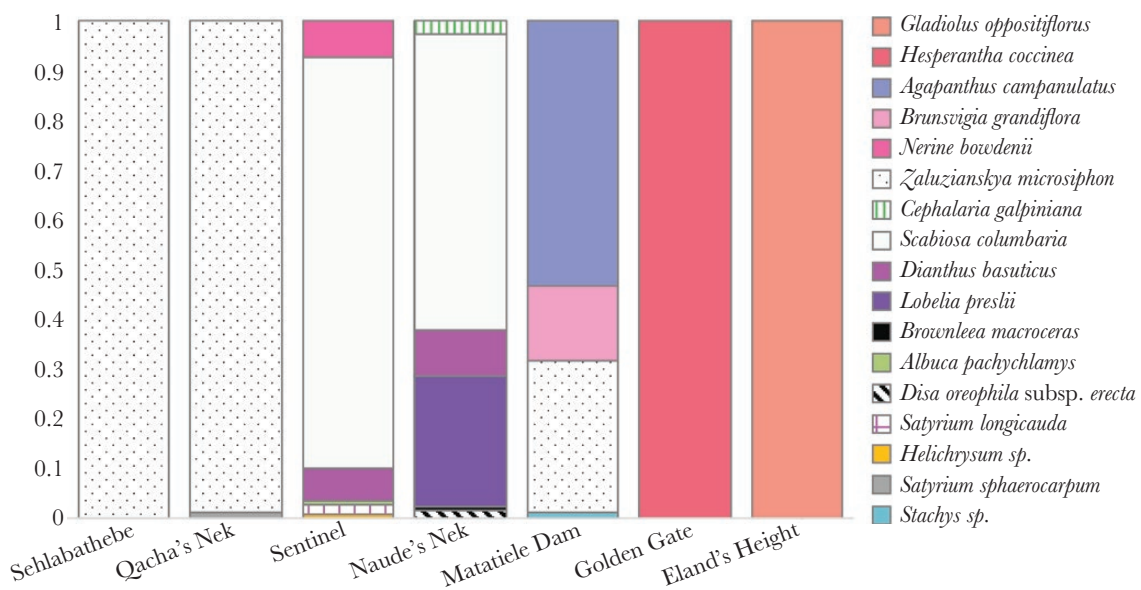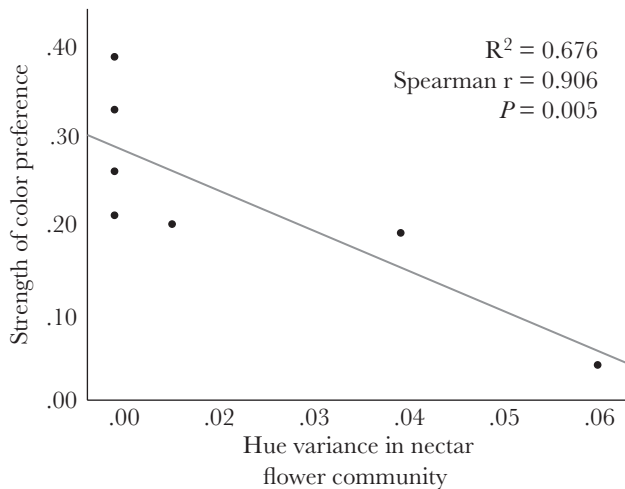

Figure 2
Flower visits by *Prosoeca ganglbaueri* at 7 sites. Proportions are as a total of observed flower visits before and after interview bouquet trials, and colors are approximate to their perception in human vision.
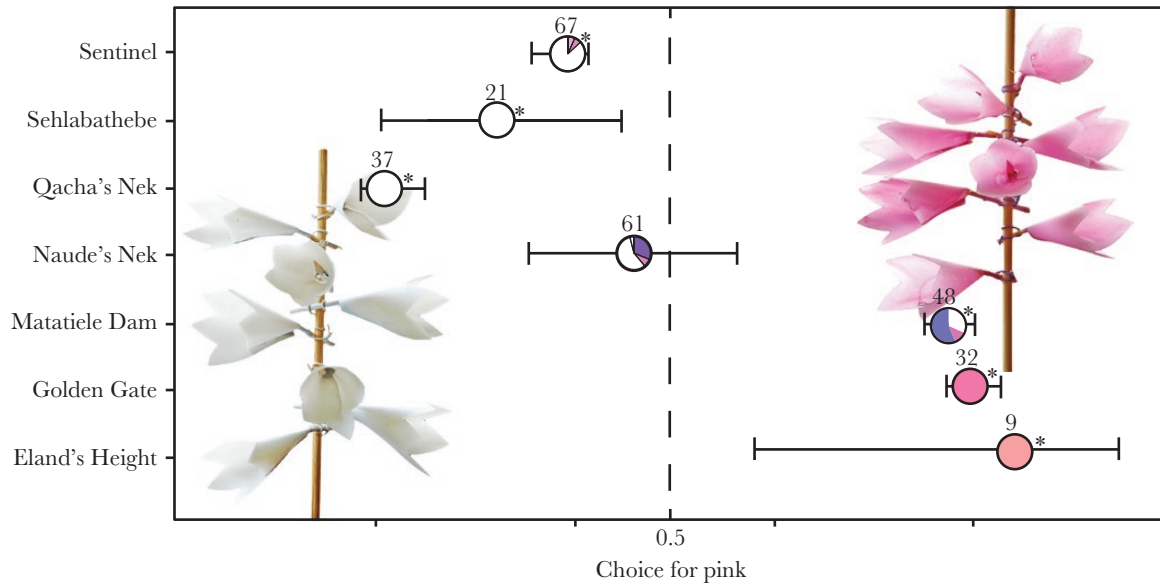
Figure 3
Binary choice (as measured by inspection or probing) for pink or white artificial flowers in *Prosoeca ganglbaueri*. Seven sites are represented on the *y* axis, each point is color-coded by proportional representation of nectar flower visits at that site (Figure 2) and appears with number of choice trials recorded and 95% confidence interval. Significant departure from chance choice denoted with asterisk.
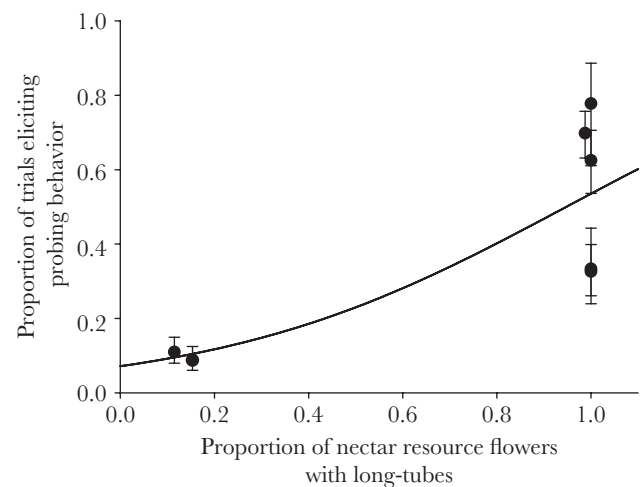

Figure 4
Correlation of absolute strength of color preference in foraging *Prosoeca ganglbaueri* with the variance in nectar resource flower hue at 7 sites.
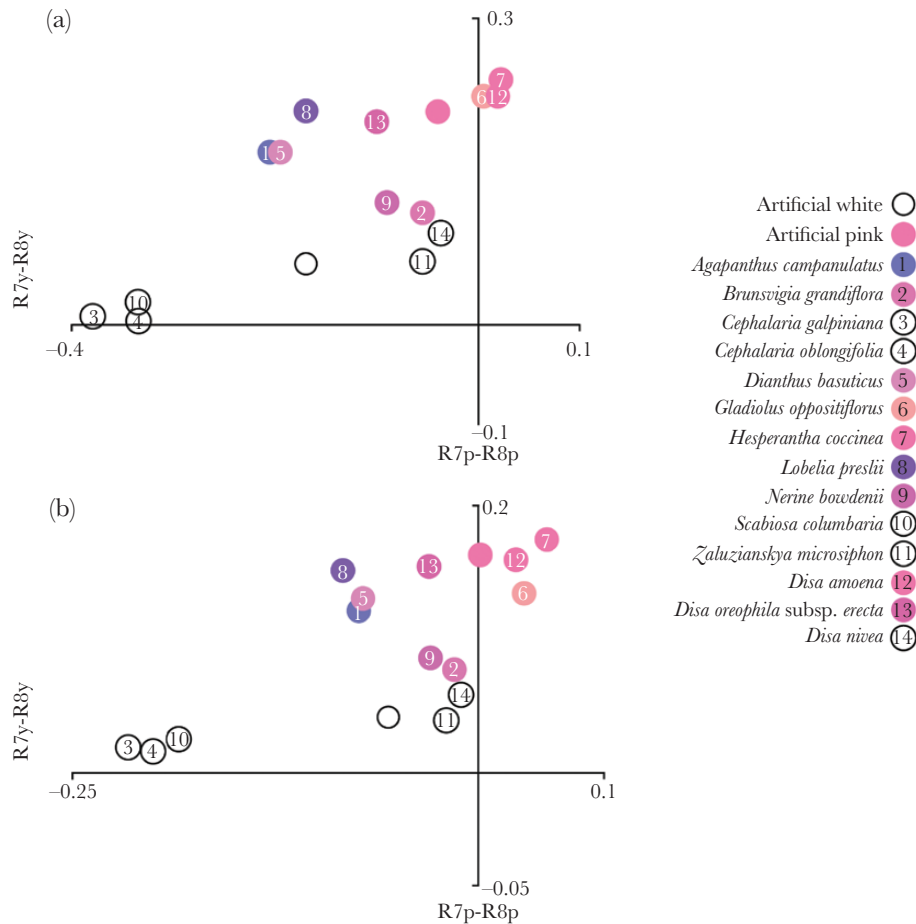

Figure 5
Probing response of *Prosoeca ganglbaueri* to artificial flowers presented in 7 plant communities. Sites varied in the proportion of long-tube to short-tube flowers used as nectar source by *P. ganglbaueri*.

were substantially more likely to probe the interview bouquet after feeding on a long-tubed flower (Supplementary Figure S2), likelihood-ratio $\chi^2 = 12.236$, $P = 0.000$. We found no similar influence of prior flower morphology on flies' choice for pink (Supplementary Figure S2), likelihood-ratio $\chi^2 = 0.171$, $P = 0.680$.

Fly vision models

For Troje's COC fly-vision model, most floral spectra fell in the upper-left quadrant, whether modeled with receptor sensitivities from *Lucilia* (Figure 6a, Supplementary Figure S3a) or *Eristalis* (Figure 6b, Supplementary Figure S3b). The exceptions were the human-perceived white or near-white flowers of *S. columbaria* and *Cephalaria* species which fell in the lower left quadrant under standard green leaf background, and the pink *Hesperantha*, *Disa amoena*, and *Gladiolus* which fell in the top-right quadrant using the grassland

habitat background. Loci for the artificial interview bouquets plotted in the upper-left quadrant of fly vision space for most combinations of model assumptions, with the exception being *Eristalis* receptor sensitivities combined with the grassland habitat background, which plotted the pink bouquet in the upper-right quadrant (Figure 6b).

Results from the Vorobyev & Osorio receptor noise limited model suggest that the artificial flowers were detectably different in color from the natural flowers: the pink and white artificial flowers all had JND values > 1 when compared to the pink, white and blue natural flowers (Supplementary Figure S4). This is also apparent in the loci of the artificial and natural flowers as plotted in the tetrahedral fly vision color space analysis (Supplementary Figure S5). The natural pink flowers clustered separately from the white and blue flowers for all the principal components generated from the

Figure 6

Colors of artificial flowers and nectar-bearing flowers visited by *Prosoeca ganglbaueri* modeled in the categorical fly visual space using (a) *Lucilia* receptor sensitivities, and (b) *Eristalis* receptor sensitivities with adaptation for grassland green background. Loci are color-coded approximate to human vision. Under this model, color loci in different quadrants cannot be discriminated.

loci (*P* values < 0.05), whereas the white and blue flowers clustered separately for PC2 (explaining 35.4% of the variation; *P* values < 0.05), but together for PC1 (explaining 62.6% of the variation; *P* values < 0.05). The pink and white artificial flower colors did not cluster with any of the natural flower colors for PC1 (all *P* values > 0.05). For PC2, pink artificial flowers clustered with white natural flowers, and white artificial flowers with blue natural flowers (*P*-values < 0.05).

DISCUSSION

Our experiment simulated visitation to Batesian floral mimics of 2 color phenotypes across different populations of pollinators. By measuring visits to artificial flowers in differing floral communities, we discovered strong site-specific color preferences in *P. ganglbaueri*. The geographic variation in color preference revealed here demonstrates that mimic signal–receiver response can be dependent on the context of the community and supports the contention that deceptive orchids pollinated by *P. ganglbaueri* are likely Batesian mimics.

Floral community color variation and pollinator color preference

There was wide among-population variation in the floral spectral reflectances of the natural nectar resources. Some sites were

characterized by only a single color of nectar-source flower, while others were composed of a wider diversity in floral color (Figure 2, Table 1). At sites where flies were observed taking nectar from flowers of only a single color (Sehlabathebe, Qacha's Nek, Golden Gate, Eland's Height), that color predicted the pink–white choice of those flies—a result consistent with the operation of conditioned preference (Figure 3).

We also found that flies at the site with highest variance in nectar source flower hue showed ambiguous pink–white preference (Figures 3 and 4). This suggests that flies feeding on a wide range of nectar sources show more generalized preferences than flies with a diet from a more limited floral color palette. At the Matatiele Dam site, we observed flies feeding from a wide variance in nectar-resource hue (Table 1), and this was the only site where blue flowers were being visited with high frequency (Figure 2). Interestingly, pink preference was exhibited at this site, and this has 2 possible explanations. First, blue might be indistinguishable from pink in *P. ganglbaueri* vision. Supporting this, the locus of blue *A. campanulatus* plots very close to the pink species *D. basuticus* in fly vision space (Figure 6, Supplementary Figure S3). Also, the reflectance spectra of these pink and blue flowers differed most substantially in the long wavelength 550–600 (green–yellow) range that may be irrelevant to foraging decisions in this species; in the field, *P. ganglbaueri* did not probe any yellow flowers, despite numerous yellow *Asteraceae*

being present at some sites. A second explanation is that preference for blue can, in the absence of blue, be generalized to pink because they share closer spectral similarity than do blue and white. Vision models were not unanimous in their position of pink flower loci, such that the COC model that best explains the pink–white preference behavior we observed (Figure 6b, discussed in more detail below) contradicts this explanation, while other conditions of the COC model support it by placing blue closer to pink than to white, all in the upper-left quadrant (Figure 6a, Supplementary Figure S3). Although our RNL color space modeling indicated that the blue flowers clustered more closely to the white than to the pink flowers, generalization of color preference is exhibited in studies with *Apis mellifera* (Dyer and Murphy 2009) and *Bombus terrestris* (Gumbert 2000), taxa for which well-validated color vision models are available. Perhaps the most relevant prior work showed that long-proboscid *Philoliche aethiopica* consistently preferred blue over pink, even if tested at a site where it fed on pink flowers (Jersakova et al. 2012). Tests like these, examining blue–pink preference at sites with varying abundances of natural blue flowers are needed to understand whether similar generalization occurs in *P. ganglbaueri*.

The color preference measured here covaried with community floral color variance in a manner consistent with conditioning, i.e., learned color preferences. We observed that at sites with a wider variety of hues associated with nectar resource plants, resident *P. ganglbaueri* displayed a more generalized color preference (Figure 4). This could be evidence that flies which use a wider range of hues show a weaker preference for any one particular color, or that flies' learning is sufficiently flexible that they can be conditioned to multiple colors. Conclusive evidence of learned preference will require measuring innate color preference in naïve flies and/or controlled training experiments which will only be possible when methods are developed for laboratory culture and husbandry of *P. ganglbaueri*.

Morphology in floral communities and pollinator preference

Flies were substantially more likely to probe the interview bouquets after feeding from a long-tubed flower, rather than a short-tubed flower (Supplementary Figure S2). The increased probing of our interview bouquets by flies using long-tube nectar flowers was also evident in the rates of probing behavior across sites in relation with the extent to which long-tubed flowers formed the nectar diet of a community's resident flies (Figure 5). As the artificial flowers are long-tubed, this result indicates a possible conditioned preference for floral morphology influencing the attractiveness of our interview bouquets. Previous work in *Prosoeca* showed that flies do not visit mimic orchids that have been artificially rearranged from the capitulum morphology normally displayed in mimic and model to an experimental raceme arrangement (Johnson et al. 2003). Flies in our study could be similarly discriminating on the basis of inflorescence architecture, which differed between short-tubed flowers (e.g., *S. columbaria*) and our bouquets, or on the perceived length of floral tubes. These results confirm that in addition to color, signal–reward associations in morphological traits also play a critical role in the exploitation of mutualisms by Batesian floral mimics.

While the morphology of the local nectar resources influenced attractiveness of our interview bouquets, any preference for morphology was seemingly disconnected from preference for color. At both Sentinel and Naude's Nek, the morphology of the flower being visited immediately prior to each choice trial showed no influence on the pink–white preference measured (Supplementary Figure S2).

Implications for floral evolution and Batesian mimicry

P. ganglbaueri is the sole pollinator of several deceptive orchid species (Anderson et al. 2005; Anderson and Johnson 2009), and the wider guild of long-proboscid flies pollinates several other putatively Batesian mimic orchids (Goldblatt and Manning 2000; Johnson 2000; Johnson et al. 2003; Johnson and Morita 2006; de Jager et al. 2016). Across our study sites, choices among artificial flowers were correlated with the color of flowers most often used as a nectar resource by resident flies, while the intensity of the behavioral response was influenced by the morphological match of our models to the nectar resources being used (Figure 5). If one views the use of artificial flowers in this study as a simulated introduction of mimic variation, and treats visits as a proxy for fitness, our results support the existence of conditions necessary for the evolution of Batesian mimicry. Importantly, the strong divergence in preferences of flies among sites is not consistent with the flies being attracted to the interview bouquets via generalized food deception.

Spectral reflectance and vision modeling for 3 species of deceptive orchid known to be pollinated by *P. ganglbaueri* also supports the operation of Batesian mimicry in the *P. ganglbaueri* pollination guild. While not observed in this study, *D. nivea* has been shown to occur only in populations of *Z. microsiphon*, where its floral traits are closely correlated to those of this nectar-bearing model (Anderson et al. 2005). Our spectral data replicated these earlier findings, and by plotting loci for both model and mimic adjacent in fly vision space (Figure 6), the vision models strongly support the hypothesis that *D. nivea* is a Batesian mimic of *Z. microsiphon*. While *D. amoena* occurs outside the area of this study, but within the distribution range of *P. ganglbaueri*, we reported spectral reflectance data and vision modeling for this species as it is a strong candidate for Batesian mimicry. The spectral similarity (Supplementary Figure S1) and vision modeling (Figure 6) showed similarity to pink species that are known to be pollinated by *P. ganglbaueri*, such as *G. oppositiflorus* and *H. coccinea*. Populations of flies feeding on *G. oppositiflorus* and *H. coccinea* in this study showed very strong pink preference (Figure 3), which suggests that *D. amoena* might be likely to be pollinated if it occurred in these populations. *D. amoena* occurs only in populations with pink *Watsonia wilmsii* and further assessment of its status as a Batesian mimic awaits analysis of floral trait data from that species. The only deceptive orchid known to be pollinated by *P. ganglbaueri* that was observed in this study is *D. oreophila* subsp. *erecta* occurring at Naude's Nek. Here, we found flies feeding on a wide range of nectar-producing species and an associated wide range of floral colors (Figure 2, Table 1). Reflectance spectra for this orchid were most similar to pink *Dianthus basuticus* with which it co-occurs, and in fly vision space its locus plotted in the upper-left quadrant along with most other flowers visited by *P. ganglbaueri*. The occurrence of this species at a site where we measured no preference, and found no apparent single model species, supports the hypothesis that instead of Batesian mimicry, *D. oreophila* subsp. *erecta* has evolved a more generalized form of food deception (Johnson and Steiner 1995).

While the behavioral evidence is strongly supportive of flexible floral color preferences in response to conditioning, we cannot rule out the alternative hypothesis that among-population variance in color preference represents fine-scale innate preference, or local adaptation, rather than local conditioning. There are 2 lines of evidence against this explanation. First, we tested and failed to find a

correlation between population pairwise distance and color preference. If preference was genetically determined, nearby populations should show correlated preference, as they are more likely to experience gene flow. On the other hand, strong selection on genetically determined preference could maintain divergent phenotypes in the face of gene flow. Second, as in other fly species, we have no reason to doubt that *P. ganglbaueri* shouldn't also be very capable of learning (Nelson 1971; Quinn et al. 1974; Fukushi 1976). Despite this, it is likely that naïve flies do have some color preferences and further study is required to understand geographic variation in naïve preference, the scale of gene flow, and potential for local adaptation.

Fly behavior and vision model performance

The clear preference for pink and white displayed at some sites confirms that *P. ganglbaueri* is capable of distinguishing between these colors. The only vision model capable of explaining this behavior was the COC model (Troje 1993) incorporating *Eristalis* receptor sensitivities (Lunau 2014; Shrestha et al. 2016) and adaptation to the grassland habitat green background (Figure 6b). Other combinations of COC model conditions placed loci for the 2 artificial bouquets in the same quadrant, inferring they would indistinguishable. Similarly, the receptor noise limited tetrachromat model (Vorobyev and Osorio 1998) found the pink artificial flowers were more similar to white and blue, rather than pink natural flowers. However, we found strong preference for pink (but not white) artificial flowers at sites where flies fed predominantly from pink natural flowers, suggesting poor performance of this model.

While the COC model produced results that fit best with observed behavior, its conclusions were contingent on the choice of which spectra were chosen for background adaptation. The diversity and complexity of backgrounds in nature therefore presents a considerable challenge to our current techniques for modeling animal vision (Bukovac et al. 2017). While its performance best reflected behavior, the *Eristalis* and grassland background COC model was still not entirely consistent with our behavioral evidence, as flies at the Matatiele Dam site showed pink preference despite most of their visits being recorded to blue and white species with loci in the upper-left quadrant (*A. campanulatus*, and *Z. microsiphon*). As discussed above, if this is explained by blue being indistinguishable from, or more similar to pink than white in fly vision, this is not reflected in the model. In addition, loci for several pink flowers (e.g., *N. bowdenii* and *D. basuticus*) fell in the upper-left quadrant, together with white and blue flowers. So, no model here fully fits with observed behavior.

Currently, these are the only available color vision models for flies, but they have both been tested against the behavior of only one species, using lab populations (*Lucilia cuprina* (Calliphoridae) (Fukushi 1994); and *Drosophila melanogaster* (Brembs and de Ibarra 2006), both of which are phylogenetically distant to *P. ganglbaueri*. The models have been applied with mixed success to various flies (Empididae, Mycetophilidae, Syrphidae, Tabanidae (Jersáková et al. 2012; Kelly and Gaskett 2014; Ohashi et al. 2015; Bodley et al. 2016; Shrestha et al. 2016)), but conflicting results and a lack of field behavioral data clearly caution against broad application of these models. A further issue is the lack of available spectral sensitivity data for diverse fly taxa. In the Hymenoptera, spectral sensitivities are highly conserved (Peitsch et al. 1992) and model honeybee data has been widely applied to other Hymenoptera. Generalization of vision models across wide taxonomic boundaries may not be suitable for Diptera, and even in Hymenoptera this practice is potentially problematic (Dyer et al. 2008). Further electrophysiological and perception experiments are therefore required to produce a more accurate working model of color vision in *P. ganglbaueri*. In particular, preference experiments to probe discrimination limits within a narrower spectral range, as well as manipulating properties such as saturation (Spaethe et al. 2014) will be required to further validate or refine the available models for color vision in *P. ganglbaueri*. Until further work shows otherwise, in the context of closer phylogenetic affinity to *Eristalis* than *Lucilia* (Wiegmann et al. 2011), their shared ecology as pollinators, and behavioral evidence here, we recommend *Eristalis* receptor sensitivities paired with local grassland background spectra to most accurately model vision in *P. ganglbaueri*, and even then conclusions should be treated with caution.

In conclusion, we have shown that the specialist nectar-feeder and keystone pollinator *P. ganglbaueri* displays a flexible floral color preference that responds predictably to local signal–reward associations. Both the color and morphology of local nectar sources strongly influenced the foraging response of flies to the experimental interview bouquets, and preferences for color and morphology varied independently of one another. This evidence is overall consistent with learning and supports the hypothesis that rewardless orchids pollinated by *P. ganglbaueri* are true Batesian mimics that have evolved signal imitation traits that exploit mutualistic relationships between fly pollinators and their surrounding floral communities.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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