

# From the butterfly's point of view: learned colour association determines differential pollination of two co-occurring mock verbains by *Agraulis vanillae* (Nymphalidae)

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Learning plays an important role in the location and utilization of nectar sources for pollinators. In this work we focus on the plant-pollinator interaction between the butterfly *Agraulis vanillae* (Nymphalidae) and two *Glandularia* plant species (Verbenaceae) that grow in sympatry. Bioassays using arrays of artificial flowers (red vs. lilac-purple) showed that naïve *A. vanillae* butterflies do not have innate colour preferences for any of the tested colours. Trained butterflies were able to learn to associate both floral colours with the presence of nectar rewards. Wild *A. vanillae* butterflies visited the red flowers of *Glandularia peruviana* much more frequently than the lilac-purple flowers of *Glandularia venturii*. Standing nectar crop measurements showed that *G. peruviana* flowers offered three times more sucrose than the flowers of *G. venturii*. Analyses confirmed that corolla colour of *G. peruviana* (red flowers) and *G. venturii* (lilac-purple flowers) were discriminable in the butterfly's colour space. These findings may indicate flexibility in *A. vanillae* preferences due to a learned association between red coloration and higher nectar rewards.

ADDITIONAL KEYWORDS: butterfly – colour preferences – colour vision – learning – Nymphalidae – pollination.

## INTRODUCTION

Flowers announce the presence of rewards through a combination of sensory signals which can evoke innate or learned pollinator behaviours that mediate effective pollination. Pollinators show distinctive and remarkable cognitive abilities such as the associative learning between visual, olfactory, electrical and/or acoustic stimuli and rewards, as well as long-lasting spatio-temporal memory of the availability, location and identity of floral reward sources (Chittka & Thomson, 2001; Schaefer & Ruxton, 2011; Clarke *et al.*, 2013). Thus, pollinator preferences can strongly influence floral trait evolution such as colour, morphology and

fragrance. Several groups of diurnal pollinators mostly rely on visual stimuli to locate rewarding flowers. In particular, it is well known that butterflies mainly rely on innate and learned colour preferences to find nectar sources and host plants (Ômura & Honda, 2005; Cepero *et al.*, 2015; Kinoshita *et al.*, 2017).

Butterflies colour vision appears to have evolved from an ancestral trichromatic system based on ultraviolet (UV)-, blue-, and green-sensitive photoreceptors. Some species also contain a fourth or fifth receptor type, related in amino acid sequence to green-sensitive insect opsins, but red shifted in absorbance (Briscoe, 2008). The suite of photoreceptor sensitivities varies extensively between butterfly families, genera, species, and sexes within a species (Arikawa *et al.*, 2005; Frentiu & Briscoe, 2008; Chen *et al.*, 2016; McCulloch *et al.*, 2016).

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The ability of a given butterfly species to perceive and discriminate colours can be revealed by its innate colour preferences and its ability to associatively learn different colours with salient cues in the environment (Blackiston *et al.*, 2011). Many butterfly species have true colour vision, i.e. they can discriminate colours based on spectral composition, regardless of their intensities (Kelber & Pfaff, 1999; Kinoshita *et al.*, 1999; Zaccardi *et al.*, 2006; Sison-Mangus *et al.*, 2008). Innate sensory preferences provide a search image to inexperienced butterflies that facilitate flower detection in dynamic environments during their first foraging bouts (Lunau & Maier, 1995). After the first flower recognition, associative learning comes into play for subsequent colour selection (Weiss, 1997). Several studies have shown that butterflies can associate different corolla colours with sugar rewards and they rapidly learn to select the rewarding colour when different colours are offered simultaneously (Swihart, 1971; Lewis & Lipani, 1990; Weiss, 1995; Kinoshita *et al.*, 1999; Rodrigues *et al.*, 2010; Blackiston *et al.*, 2011). Thus, cognitive abilities allow butterflies to take advantage of a given floral source when they are able to choose among a variety of plant species that differ in their stimuli and profitability in the wild (Chittka & Thomson, 2001).

The ability to modulate colour preferences would allow the butterflies to adjust flower foraging in spatially and temporally changing community scenarios. Despite the importance of behavioural biases and abilities of butterflies in resource recognition (e.g. Weiss & Papaj, 2003; Briggs *et al.*, 2018) few studies have linked colour perception and flower choice experimental findings with those that actually occur in the wild. Moreover, wild lepidopterans are well-suited for investigations of colour preference behaviour in a natural scenario because they are important but understudied pollinators (Rader *et al.*, 2016). In this study we focus on experimental evidence for innate and learned colour preferences of the butterfly *Agraulis vanillae maculosa* (Stichel, 1908) (Nymphalidae) and its pollination interaction in the wild with two sympatric *Glandularia* J. F. Gmel. species (Verbenaceae) that differ in corolla coloration. Innate and learned colour preferences have been studied in laboratory conditions or in the wild for some Nymphalidae and Papilionidae butterfly genera (Swihart & Swihart, 1970; Rodrigues *et al.*, 2010; Blackiston *et al.*, 2011). For *A. vanillae*, experiments with real flowers strongly suggest that this species has the ability to associate flower colour with reward (Weiss, 1991). However, studies of innate colour bias and associative colour learning with artificial flowers has to our knowledge not been carried out for *A. vanillae*. The use of artificial flowers eliminates potential biases due to flower cues other than colour, such as odour, taste or shape, that may also be associatively learned

and could produce, when integrated with colour cues, spurious association with colour.

In the present work we address the following questions:

- (1) Does *A. vanillae* have innate preferences for any of the tested flower colours?
- (2) Does this butterfly, free from other biases, learn to associate flower colour with nectar reward?
- (3) Do these behavioural abilities, such as possible learned associations with reward quantity, help to explain differential visitation rates of *A. vanillae* to sympatric *Glandularia* species that differ in corolla coloration?
- (4) Are the flowers of the *Glandularia* species discriminable in the colour space of a model butterfly?

## MATERIAL AND METHODS

### STUDY SYSTEM

#### *Glandularia* species (Verbenaceae)

The present study is focused on two butterfly-pollinated, self-incompatible and pollinator-dependent *Glandularia* species from the western Chaco woodlands in central Argentina, red-flowered *G. peruviana* (L.) and lilac-purple-flowered *G. venturii* (Moldenke) Botta (Imhof *et al.*, 2013; Aguirre LA, Drewniak ME & Moré M, pers. comm.). These two species are very common in the western Chaco woodlands and have a long-lasting flowering period from October to March. Herbarium specimens (MED5-MED6 for *G. peruviana* and MED3-MED4 for *G. venturii*) were deposited at “Museo Botánico de la Universidad Nacional de Córdoba”, Argentina (CORD).

#### *Agraulis vanillae maculosa* (Nymphalidae: Heliconiinae)

*A. vanillae* occurs across the New World, from the northern United States to central Argentina, associated with the availability of several species of *Passiflora* L. (Passifloraceae), its principal larval food source (Lamas, 2004). The subspecies *A. vanillae maculosa* occurs in open environments in southern South America and is a frequent flower visitor of several native plant species (Fuhro *et al.*, 2010).

### EXPERIMENTAL SET-UP

#### *Study taxa*

Naïve *A. vanillae maculosa* butterflies were collected as caterpillars on vines of *Passiflora caerulea* L. in

the surroundings of the city of Córdoba (Córdoba, Argentina). Larvae were fed with fresh *P. caerulea* leaves and pupae were kept in cages until the emergence of imagoes. Later, adults were marked on the underside of the hindwings with indelible ink pen of different colours for individual identification. Males and females were kept in separate cages.

### Artificial flowers

Bioassays using arrays of artificial flowers of two different colours (red vs. lilac-purple) were undertaken to investigate both innate colour preferences and colour learning in *A. vanillae*. Artificial flowers were used to decouple visual from olfactory signals, since the two *Glandularia* species studied here differ in their chemical composition of floral fragrances (Moré M. and Raguso RA., pers. comm.). Thus, artificial flowers were made of scentless synthetic fabric and 200  $\mu$ L plastic tubes. Artificial flowers showed a secondary peak in the UV region that was absent in the corolla reflectance spectra from natural flowers. Flowers were similar in shape to those of *Glandularia* with an area of 4.77 cm<sup>2</sup> and a diameter of 2.5 cm.

### Testing arena

The testing arena consisted of 12 artificial flowers, six of each colour (red and lilac-purple), arranged in a 3  $\times$  4 grid. Artificial flowers were placed 9 cm apart from each other (distance measured from centre to centre) against a green background inside a wire mesh field cage of 0.3  $\times$  0.3  $\times$  0.3 m in size (Fig. 1B). Flower positions were changed within the array after

each trial. Bioassays were performed on sunny days from 10:00 to 15:00 during two consecutive flowering seasons (November 2017 - March 2018 and December 2018 - March 2019) inside a greenhouse under natural illumination lacking UV light (i.e. polycarbonate ceiling filtered UV light; [Supporting Information, Fig. S2](#)).

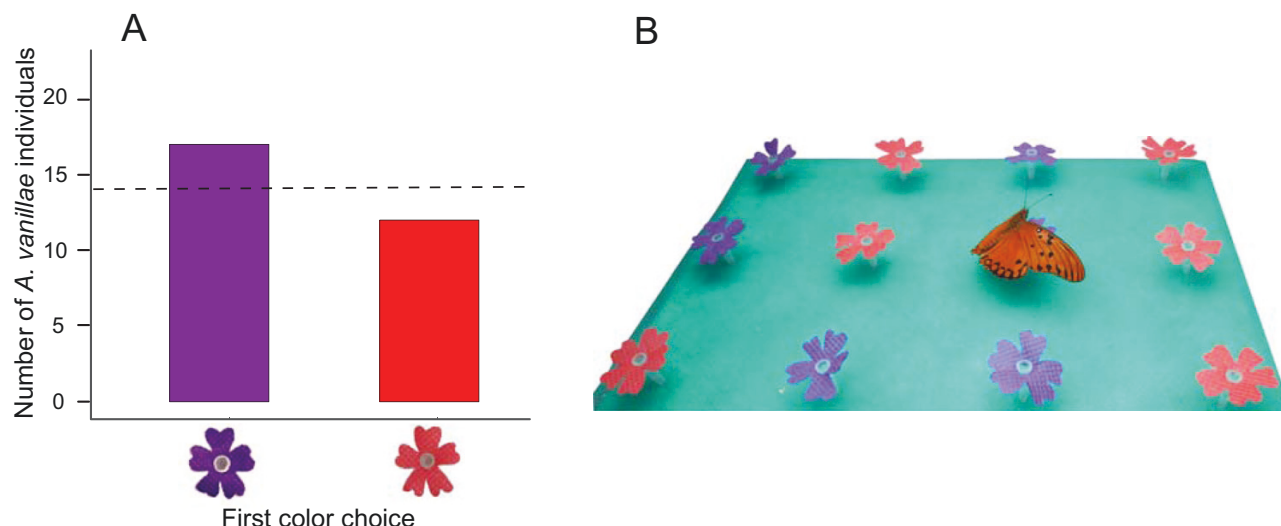
To assure that each butterfly had previously experienced a nectar stimulus, we unrolled the butterfly proboscis using an entomology pin into a solution of 30% sucrose in water offered in a 200  $\mu$ L transparent plastic tube. A previous study suggests that this concentration is the optimal for *A. vanillae* (May, 1985). Each butterfly was fed for 5 s before it was released into the testing arena.

### Innate colour preferences

Each butterfly was individually tested and the colour of the first flower visited was recorded. A visit was recorded only if the butterfly landed on the corolla and extended its proboscis into the tube. Colour choices of naïve butterflies were analysed using G tests. A total of 59 butterflies were tested (20 females and 39 males) and 29 butterflies responded to the tests (12 females and 17 males). A total of 20 butterflies did not respond and were discarded. Because innate colour preferences of *A. vanillae* did not differ among females and males, individuals were pooled.

### Associative colour learning

*A. vanillae* adults were trained to visit either red or lilac-purple artificial flowers. Only flowers of the training colour were rewarded with a solution of 30%



**Figure 1.** Innate colour preferences bioassays using artificial flowers. A, *A. vanillae* butterflies did not show significant deviations from random choice in lilac-purple and red artificial flowers. The dotted line shows the expected frequency for a random choice ( $N = 29$  individuals). B, testing arena.

sucrose in water. Each training session lasted 2 h, and only one training session was done each day. Each butterfly was individually tested for colour choice after at least three previous training sessions and 2 h after the last training.

Those butterflies that did not visit a flower after 10 min during testing were trained again the following day, and tested again after 2 h of the latest training session. Before each trial butterflies were fed using the artificial flower of the training colour for 5 s to stimulate them to forage for nectar. This standard procedure is used in bioassay testing for lepidopteran preferences (e.g. [Rodrigues et al., 2010](#); [Blackiston et al., 2011](#); [Cepero et al., 2015](#); [Ramos et al., 2019](#)). Butterflies were released individually into the testing arena over a 10 min period and the colour of the first visited flower (i.e. landing and proboscis extension) was recorded and these individuals were discarded. Colour choices of trained butterflies were analysed using G tests. A total of 55 butterflies (20 females and 35 males) were trained in the testing arena in groups of five to eight individuals of the same sex during two to six training sessions. A total of 39 butterflies responded to the tests (18 females and 21 males). A total of 16 butterflies did not respond and were discarded. As learned colour preferences of *A. vanillae* did not differ among females and males, individuals were pooled.

#### *Visitation rate in a natural community*

Observations were carried out in the “Reserva Hídrica, Natural y Recreativa Bamba”, La Calera, Córdoba province, Argentina. Pollinator observations were recorded simultaneously for the two *Glandularia* species once a week throughout the flowering season, from October to March for two consecutive seasons (2017–2019). A total of 52 observation periods of 10 min, 27 in *G. peruviana* and 25 in *G. venturii*, were performed between 10:00 to 17:00 during *A. vanillae* peak activity ([Drewniak et al., 2016](#)). The number of flower visits by *A. vanillae* individuals and the total number of flowers observed were recorded. The visits were recorded only if the butterfly landed on the corolla and extended its proboscis to obtain nectar from the flowers. Visitation rate was calculated as the number of visits per flower per hour. Differences in visitation rates among *Glandularia* species in the wild were examined using a generalised linear model (GLM) with a log-link and Poisson error structure. Since overdispersion was detected in the model, the error structure was corrected using a quasi-GLM ([Agresti, 2015](#)). Calculations were performed using the *lme4* package ([Bates et al., 2015](#)) of R software ([R Core Team, 2019](#)).

#### COROLLA COLOUR AND NECTAR REWARD VARIATION BETWEEN *GLANDULARIA* SPECIES

##### *Spectra processing and visual modeling*

Corolla reflectance of the two focal *Glandularia* species (*G. peruviana* and *G. venturii*) and the artificial flowers (red and lilac-purple) used in the bioassays was measured using a USB4000 spectrophotometer equipped with a pulsed xenon light source with a light emission range between 220 and 750 nm, and a UV-visible reflection/backscatter probe that comprises high hydroxyl UV-visible fibres with a wavelength range between 300–1100 nm (Ocean Optics Inc., Dunedin, FL, USA). The corolla-reflected light was collected at 45° to avoid specular reflectance. The probe was mounted in a prismatic holder and the distance to the corolla surface was 1 mm. A white standard (WS-1-SS White Standard, Ocean Optics Inc.) was used to re-calibrate the equipment between each measurement in order to correct for possible shifts in spectrophotometer performance ([Chittka & Kevan, 2005](#)). SpectraSuite software was used to collect three individual scans, with a boxcar width of 100 nm and an integration time of 2 s per scan. A total of two flowers per individual and 28 individuals per species were measured. Processing and visualization of the reflectance spectra were done using the *pavo 2.2.0* package ([Maia et al., 2019](#)) of R software ([R Core Team, 2019](#)). Spectra were trimmed to the insect vision range (300–700 nm) using the *as.rspec* function. Spectra were smoothed and negative reflectance values, which can be introduced by electrical noise in the spectrophotometer, were removed using the *procspec* function with span set to 0.25. Measurements were averaged by individual and within species using the *aggspec* function.

Two complementary approaches were used to assess corolla colour of *Glandularia* species. First, we performed a Principal Component Analysis (PCA) to examine overall differences in raw reflectance spectra, which makes no assumptions about the receiver's visual system. Spectra were standardized to have a mean reflectance of zero (i.e. removing luminance as a dominant variable in the PCA) so they only represent spectral shape ([Cuthill et al., 1999](#)) and overall variation was represented in a biplot ([van der Kooi et al., 2016](#)). Second, we represented the reflectance spectra of natural and artificial flowers in the visual perceptual space of a model butterfly with three photoreceptors to examine how these differences among corolla reflectance might be perceived by actual pollinators. We use the *pavo v.2.2.0* package ([Maia et al., 2019](#)) of R software ([R Core Team, 2019](#)) to construct a ‘visual model’ ([Supporting Information, Appendix S1](#)) that gives an approximation of the perception and discrimination of a model butterfly given the parameters specified below [review by [Renoult et al. \(2017\)](#)]. The first step was to model

the spectral sensitivity with the *sensmodel* function using the photoreceptor peak sensitivities according to the models of Govardovskii *et al.* (2000) and Hart and Vorobyev (2005). The adult compound eye of *A. vanillae* expresses three opsin-encoding cDNAs: an UV, a blue and a long-wavelength (L) opsin (Yuan *et al.*, 2010). Epi-microspectrophotometric and retinal densitometry measurements indicate that *A. vanillae* butterfly eyes have a 555 (L) nm receptor (Frentiu *et al.*, 2007) and a 370 (UV) nm receptor (Yuan *et al.*, 2010), respectively. Although the wavelength of peak sensitivity (max. value) of the *A. vanillae* blue receptor has not yet been measured, in several closely-related species the blue receptor ranges from 464 to 470 nm [*Dryas iulia*, 470 nm (Yuan *et al.*, 2010); *Heliconius erato*, 470 nm (McCulloch *et al.*, 2016); *Heliconius numata*, 467 nm (Briscoe *et al.*, 2010); *Heliconius hecale*, 464 nm (Yuan *et al.*, 2010)]. Thus, we used the following maximum sensitivity peaks: 370, 470 and 555 nm. The second step was to analyse the perception of flower colour with the *vismodel* function. This function estimates photoreceptor stimulation by calculating quantum catches of each photoreceptor ( $Q_{370}$ ,  $Q_{470}$  and  $Q_{555}$ ) incorporating a von Kries transformation that normalizes receptor quantum catches to the background (Maia *et al.*, 2019). Two background reflectance spectra were utilized: the green fabric used in the bioassays for the artificial flowers and the green foliage (calculated as the mean reflectance spectra of the leaves of *G. peruviana* and *G. venturii*) for the natural flowers (Supporting Information, Fig. S2). Two alternative illuminant spectra for the *vismodel* function were utilized: D65 (standard daylight) for natural flowers, and natural illumination of the room where experiments were carried out for the artificial flowers (Supporting Information, Fig. S2). To visualize colour perception of natural and artificial flower as loci in perception space, quantum catch scores ( $Q_i$ ) were modelled in a trichromatic chromaticity space using *colspace* function. Finally, the *coldist* function was used to evaluate the discrimination between flower colours for both natural and artificial flowers. This function calculates colour distances with the Receptor Noise Limited (RNL) model of Vorobyev and Osorio (1998) with noise based on the relative densities of photoreceptors. RNL was developed to predict colour vision thresholds. One of the assumptions is that the thresholds are given by the noise that arises in the receiver's channels. The following options were utilized for the *coldist* function: Weber fraction of 0.05 as empirically estimated for several butterflies (Koshitaka *et al.*, 2008); photoreceptor densities of: UV = 0.12, B = 0.21, L = 1 (male) or UV = 0.19, B = 0.14, L = 1 (female) based on photoreceptor counts from *Eueides isabella*, another Heliconiinae butterfly with the same three opsins as *A. vanillae* (McCulloch *et al.*, 2017: Table S3, Supplementary Material online); and “qcatch”

option was set to fi which transforms quantum catches according to Fechner's law. The discriminability of two colours,  $\Delta S$ , is modelled in units of ‘Just Noticeable Differences’ (JND). Thus, discriminability among natural and artificial flowers was reported in  $\Delta S$  values of one JND. Values of  $\Delta S$  greater than one unit indicate that colours are discriminable in the visual model.

#### Nectar reward quantity

Nectar was extracted with calibrated 2  $\mu$ L and 5  $\mu$ L microcapillaries from freshly open flowers collected early in the morning before butterfly activity started. Measurements were done in five to ten flowers per individual and in ten individuals for each *Glandularia* species. Concentration was measured in sucrose equivalents (% w/w) with a temperature-compensated handheld refractometer (0–62° Brix, Atago Inc., Tokyo, Japan). Significant differences in volume and sucrose concentration among species were assessed using a mixed linear model using the *lmerTest* package (Kuznetsova *et al.*, 2017) of R software (R Core Team, 2019).

## RESULTS

### INNATE COLOUR PREFERENCES OF NAÏVE *A. VANILLAE* BUTTERFLIES TO ARTIFICIAL FLOWERS

No innate colour preferences of *A. vanillae* butterflies were detected in the dual choice test for red and lilac-purple artificial flowers ( $G = 0.55$ ,  $df = 1$ ,  $P = 0.46$ ,  $N = 29$ ; Fig. 1).

### Associative colour learning in *A. vanillae* butterflies

*A. vanillae* butterflies learned to associate each of the training colours with the presence of a sucrose reward ( $G = 11.93$ ,  $df = 1$ ,  $P < 0.001$ ,  $N = 39$ ). From the total of 55 trained individuals, 30 learned to visit the training colour, nine visited the non-rewarding colour, and 16 individuals did not respond to the stimulus. The learning process was fast, with 84% of the individuals choosing the rewarding colour within the first five training sessions (Supporting Information, Fig. S1). The proportion of butterflies that learned was slightly higher for the red (0.80) than for the lilac-purple (0.71) flowers although this difference was not statistically significant.

### Visitation rate of wild *A. vanillae* butterflies to *Glandularia* species in a natural community

Wild *A. vanillae* butterflies visited the red flowers of *G. peruviana* exclusively (study season I) or much more frequently than the lilac-purple flowers of *G. venturii*

(study season II) in the studied community according to the fitted model (quasi-generalized linear model,  $t = 2.254$ ,  $P = 0.028$  with species factor and  $t = 1.837$ ,  $P = 0.07$  with season factor; Fig. 2). During the first study season, *A. vanillae* butterflies exclusively visited *G. peruviana* flowers and *G. venturii* flowers were not visited although more than 1700 flowers were inspected (Fig. 2A; Supporting Information, Fig. S2). In the second study season, *G. peruviana* flowers were visited by *A. vanillae* twice more frequently than in the first season, and *G. venturii* flowers were visited although in a very low frequency (Fig. 2A; Supporting Information, Fig. S2).

#### Nectar reward variation between *Glandularia* species

A large difference in nectar rewards was recorded between studied species. *G. peruviana* offered three times more sucrose per flower than *G. venturii* ( $t = 5.93$ ,  $P = 5.07 \times 10^{-11}$ ; Supporting Information, Table S1).

#### Corolla colour variation between *Glandularia* species

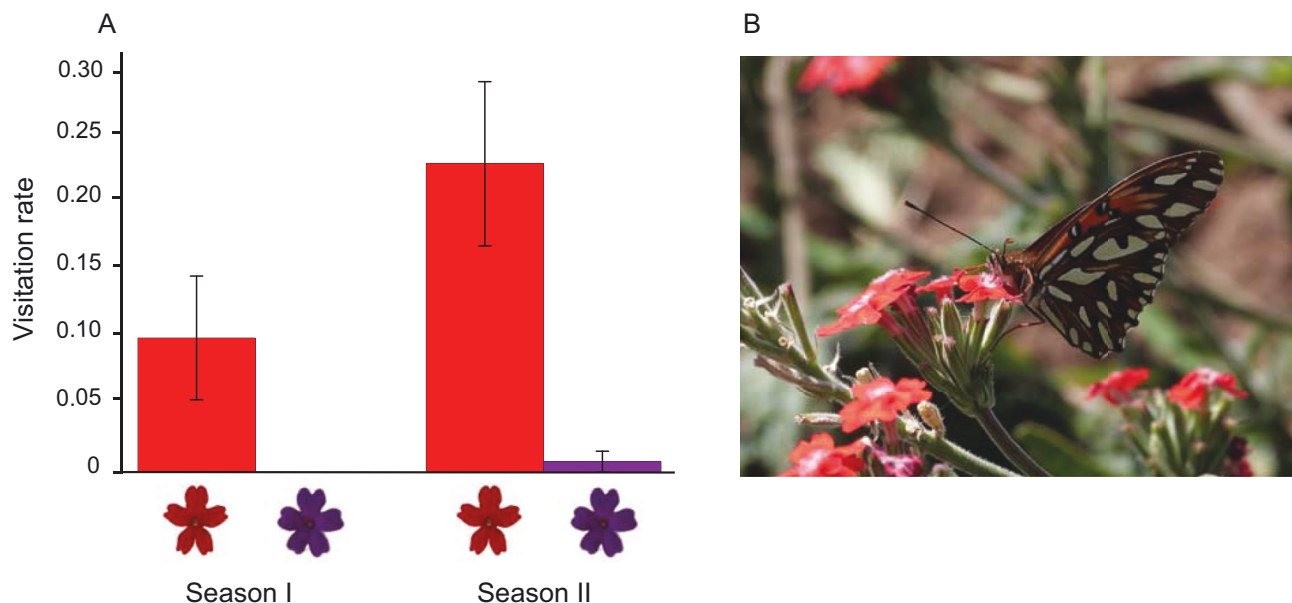
The PCA analysis showed a large variation in raw corolla reflectance spectra in both, between the two *Glandularia* species and among individuals within each species (Fig. 3). The first two principal components (PCs) accounted for 93% of total variation

in corolla reflectance spectra. PC1 represents variation in the amount of short and medium relative to long wavelengths and PC2 describes variation in short wavelengths (300–384 nm) (Fig. 3).

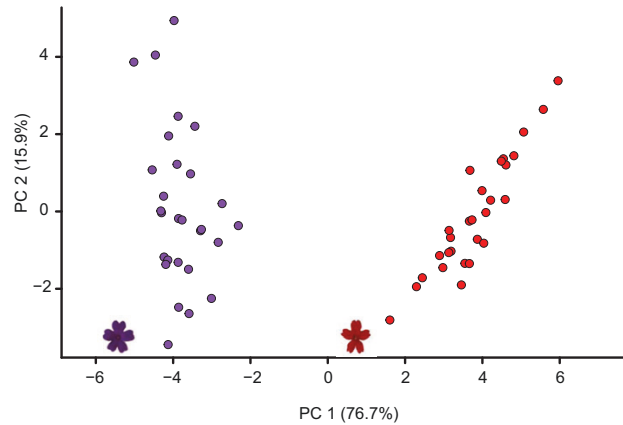
Differences among corolla reflectance between *G. peruviana* and *G. venturii* flowers on the one hand (Fig. 4A) and between red and lilac-purple artificial flowers used in the bioassays on the other (Fig. 4B), were discriminable in the visual space of the model butterfly (Fig. 4C). For natural flowers, the values exceeded the  $\Delta S$  thresholds of one JND (male  $\Delta S = 16.50$  and female  $\Delta S = 14.88$ ) using the following parameters: illumination = D65 and background = *Glandularia* leaves (Supporting Information, Fig. S3). For artificial flowers, the values also exceeded the  $\Delta S$  thresholds of one JND (male  $\Delta S = 9.30$  and female  $\Delta S = 7.89$ ) using the following parameters: illumination = greenhouse and background = green fabric (Supporting Information, Fig. S3).

## DISCUSSION

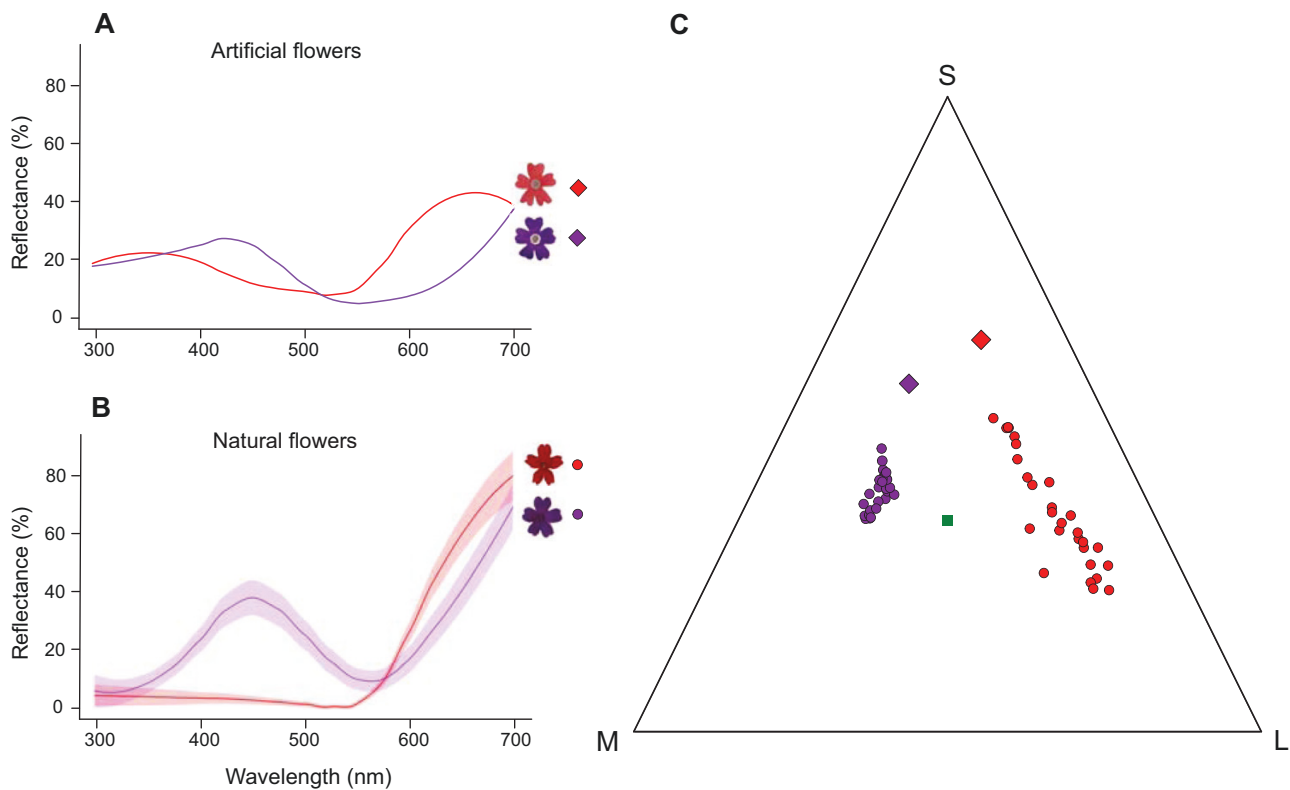
Although pollinator preferences can have important implications for the evolution of floral traits such as corolla coloration, studies that examine the consistency of pollinator behaviour in experimental arenas with those observed in the wild are rare (Briggs *et al.*, 2018). In this study we compared colour choices of the nymphalid butterfly *A. vanillae* in both



**Figure 2.** Visitation rates of wild *A. vanillae* butterflies to *G. peruviana* (red flowers) and *G. venturii* (lilac-purple flowers) in the Reserva Bamba community during two consecutive flowering seasons. A, bars represent mean number of visits per flower per hour and whiskers represent standard deviation. *Glandularia* species significantly differed in *A. vanillae* visitation rates in the two studied seasons ( $P < 0.001$ ). B, *A. vanillae* pollinating *G. peruviana* flowers.



**Figure 3.** Corolla reflectance scatterplot based on the PC1 and PC2 values of *G. peruviana* ( $N = 28$ ) and *G. venturii* ( $N = 28$ ) individuals. See text for further details.



**Figure 4.** Mean reflectance spectra of artificial and natural flowers and Maxwell's triangle of a model butterfly. A, reflectance spectra of red and lilac-purple artificial flowers used in the bioassays. B, mean reflectance spectra of *G. peruviana* (red) and *G. venturii* (lilac-purple) flowers ( $N = 28$ ). C, Maxwell's triangle of a model butterfly. Loci within the triangle represent colours exciting all three receptor types: S (short-wavelength) for UV, M (medium-wavelength) for blue and L (long-wavelength) for green. Circles show colour loci of natural flowers under D65 illumination and *Glandularia* leaves background, *G. peruviana* in red and *G. venturii* in lilac-purple. Diamonds show colour loci of artificial flowers under greenhouse illumination and green fabric background. The green square represents the green background.

artificial and natural flowers and we conclude that the observed floral choices are the result of associative learning. This approach allowed us to decouple visual from olfactory signals, since *Glandularia* species

significantly differed in their chemical composition of floral fragrances.

We did not find differences in innate preferences of *A. vanillae* for red or lilac-purple artificial flowers,

despite high chromatic contrast values. Weiss (1995) also did not find innate colour preferences when natural flowers of *Lantana camara* of two different colours (red and yellow), whose nectar has been depleted, were offered to wild *A. vanillae* butterflies. This butterfly species could have an innate preference for other colours, but this has not yet been experimentally tested. Different colours have been reported as innately preferred for many butterfly species; however, these preferences may vary according to the number and colour selection chosen in the experimental bioassays (Ilse & Vaidya, 1956; Weiss, 1997; Blackiston *et al.*, 2011).

Many behavioural studies using artificial flowers have shown that other butterfly species may have innate preferences for different colours and the ability to learn new associations between a given colour and the presence of rewards (e.g. Swihart, 1971; Scherer & Kolb, 1987; Goulson & Cory, 1993; Kelber & Pfaff, 1999; Kinoshita *et al.*, 1999; Weiss & Papaj, 2003). The lack of innate colour preferences found in our study and reported by others [e.g. Blackiston *et al.* (2011) for the nymphalid species *Danaus plexippus* and Balamurali *et al.* (2018) for several tropical bee species] is intriguing and an important result given the general knowledge that visual systems may have evolved innate colour preferences as an adaptive strategy to maximise recognition and exploitation of different nectar sources available in a given community.

Associative colour learning bioassays using artificial flowers allowed us to decouple corolla coloration from other floral traits intrinsic to natural *Glandularia* flowers such as fragrance and corolla size and shape that significantly differed among studied *Glandularia* species (Drewniak ME, pers. comm.). We confirmed that *A. vanillae* butterflies have a flexible colour learning capability; they are able to associate, within a few training sessions, artificial flowers of a given colour (red or lilac-purple) with the presence of a reward. This rapid learning rate has been previously reported for *A. vanillae* using natural flowers of *L. camara* of two different colours (Weiss, 1995). The capacity for rapid and flexible associative learning observed in *A. vanilla*, widely reported in the literature for other butterfly species, presumably allows them to adjust their foraging efforts in response to floral rewards that vary over space or time within a community (Weiss, 1997; Baracchi, 2019).

We showed that wild *A. vanillae* butterflies visited almost exclusively the red flowers of *G. peruviana* instead of the co-occurring lilac-purple flowers of *G. venturii*. Differential visitation rates of *A. vanillae* were also reported for *L. camara*, where the rewarding yellow flowers were visited more frequently than the non-rewarding red flowers (Weiss, 1991, 1995).

Corolla colour of the two sympatric *Glandularia* species and the two artificial flowers used in the bioassays were discriminable in the perceptual colour space of a model butterfly. Although artificial flowers did not have exactly the same reflectance as the natural flowers, colour loci appeared close to the ones of the natural flowers in the butterfly visual space. Although absorbance spectra for the *A. vanillae* green receptor [ $\lambda_{\text{max}} = 555 \text{ nm}$  (Frentiu *et al.*, 2007)] and UV receptor [ $\lambda_{\text{max}} = 370 \text{ nm}$  (Yuan *et al.*, 2010)] are known, the absorbance spectrum of the blue receptor and exact photoreceptor abundances not known. Our modelling outcomes are therefore approximations which nonetheless support more direct evidence such as associative learning experiments that the butterflies can indeed discriminate between the flowers.

Preferences of wild *A. vanillae* for *G. peruviana* flowers may indicate flexibility in colour preference due to learning associations between higher rewards and red coloration, since *G. peruviana* flowers offered three times more sucrose than the lilac-purple flowers of *G. venturii*. Moreover, flexibility in colour preferences of wild butterflies may be influenced by other factors that depend on the community context. These factors may include characteristics of the natural community such as the presence of other plant species with similar flowers, and the presence of competitors for the same nectar resources (Osorio & Vorobyev, 2008; Brosi & Briggs, 2013; Fornoff *et al.*, 2017). *G. peruviana* and *G. venturii* are pollinated by a wider assemblage of native butterfly species than *A. vanillae*. There are at least 14 other butterfly species visiting the two sympatric mock verbain species in the studied community: six species exclusively visited the red-flowered species *G. peruviana*, five species visited exclusively the lilac-purple-flowered *G. venturii*, and three species visited both *Glandularia* species (Drewniak ME, unpublished results). Moreover, in the studied community other red-flowered plant species such as *Zinnia peruviana* (Asteraceae) and *Oxypetalum coccineum* (Apocynaceae) also grow, and are frequently visited and pollinated by *A. vanillae* and other nymphalid species (Wiemer, 2010). Similar findings were also reported in the fynbos of South Africa for *Meneris* sp. (Nymphalidae), a major pollinator of several red-flowered plant species (Johnson & Bond, 1994). Other factors such as morphological mismatching between butterfly proboscis and corolla tube lengths, and the random chance of encounter based on plant species' relative abundances may also influence preferences of wild butterflies. Further ecological studies are needed to disentangle the role of these factors in our study system.

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## REFERENCES

- Agresti A. 2015.** *Foundations of linear and generalized linear models*. Hoboken: J. Wiley & Sons.
- Arikawa K, Wakakuwa M, Qiu X, Kurasawa M, Stavenga DG. 2005.** Sexual dimorphism of short-wavelength photoreceptors in the small white butterfly, *Pieris rapae crucivora*. *Journal of Neuroscience* **25**: 5935–5942.
- Balamurali GS, Nicholls E, Somanathan H, de Ibarra NH. 2018.** A comparative analysis of colour preferences in temperate and tropical social bees. *The Science of Nature* **105**: 8.
- Bates D, Mächler M, Bolker BM, Walker SC. 2015.** Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* **67**: 1–48.
- Baracchi D. 2019.** Cognitive ecology of pollinators and the main determinants of foraging plasticity. *Current Zoology* **65**: 421–424.
- Blackiston D, Briscoe AD, Weiss MR. 2011.** Color vision and learning in the monarch butterfly, *Danaus plexippus* (Nymphalidae). *Journal of Experimental Biology* **214**: 509–520.
- Briggs HM, Graham S, Switzer CM, Hopkins R. 2018.** Variation in context-dependent foraging behavior across pollinators. *Ecology and Evolution* **8**: 7964–7973.
- Briscoe AD. 2008.** Reconstructing the ancestral butterfly eye: focus on the opsins. *Journal of Experimental Biology* **211**: 1805–1813.
- Briscoe AD, Bybee SM, Bernard GD, Yuan F, Sison-Mangus MP, Reed RD, Warren AD, Llorente-Bousquets J, Chiao CC. 2010.** Positive selection of a duplicated UV-sensitive visual pigment coincides with wing pigment evolution in *Heliconius* butterflies. *Proceedings of the National Academy of Sciences U.S.A.* **107**: 3628–3633.
- Brosi BJ, Briggs HM. 2013.** Single pollinator species losses reduce floral fidelity and plant reproductive function. *Proceedings of the National Academy of Sciences U.S.A.* **110**: 13044–13048.
- Cepero LC, Rosenwald LC, Weiss MR. 2015.** The relative importance of flower color and shape for the foraging monarch butterfly (Lepidoptera: Nymphalidae). *Journal of Insect Behavior* **28**: 499–511.
- Chen P-J, Awata H, Matsushita A, Yang E-C, Arikawa K. 2016.** Extreme spectral richness in the eye of the common bluebottle butterfly, *Graphium sarpedon*. *Frontiers in Ecology and Evolution* **4**: 1–12.
- Chittka L, Kevan PG. 2005.** Flower colors as advertisement. In: Dafni A, Kevan PG, Husband BC, eds. *Practical pollination biology*. Cambridge: Enviroquest Ltd., 157–196.
- Chittka L, Thomson JD, eds. 2001.** *Cognitive ecology of pollination: animal behaviour and floral evolution*. Cambridge: Cambridge University Press.
- Clarke D, Whitney H, Sutton G, Robert D. 2013.** Detection and learning of floral electric fields by bumblebees. *Science* **340**: 66–69.
- Cuthill IC, Bennett AT, Partridge JC, Maier EJ. 1999.** Plumage reflectance and the objective assessment of avian sexual dichromatism. *The American Naturalist* **153**: 183–200.
- Drewniak ME, Zapata AI, Beccacece HM, Ficetti F. 2016.** Mariposas diurnas de la Reserva Natural Urbana General San Martín, Córdoba, Argentina (Lepidoptera: Papilionoidea). *SHILAP Revista de Lepidopterología* **44**: 287–298.
- Fornoff F, Klein AM, Hartig F, Benadi G, Venjakob C, Schaefer HM, Ebeling A. 2017.** Functional flower traits and their diversity drive pollinator visitation. *Oikos* **126**: 1020–1030.
- Frentiu FD, Bernard GD, Sison-Mangus MP, Van Zandt Brower A, Briscoe AD. 2007.** Gene duplication is an evolutionary mechanism for expanding spectral diversity in the long-wavelength photopigments of butterflies. *Molecular Biology and Evolution* **24**: 2016–2028.
- Frentiu FD, Briscoe AD. 2008.** A butterfly eye's view of birds. *BioEssays* **30**: 1151–1162.
- Fuhro D, Araújo AMD, Irgang BE. 2010.** Are there evidences of a complex mimicry system among *Asclepias curassavica* (Apocynaceae), *Epidendrum fulgens* (Orchidaceae), and *Lantana camara* (Verbenaceae) in Southern Brazil? *Brazilian Journal of Botany* **33**: 589–598.
- Govardovskii VI, Fyhrquist N, Reuter TOM, Kuzmin DG, Donner K. 2000.** In search of the visual pigment template. *Visual Neuroscience* **17**: 509–528.
- Goulson D, Cory JS. 1993.** Flower constancy and learning in foraging preferences of the green-veined white butterfly *Pieris napi*. *Ecological Entomology* **18**: 315–320.
- Hart NS, Vorobyev M. 2005.** Modelling oil droplet absorption spectra and spectral sensitivities of bird cone photoreceptors. *Journal of Comparative Physiology A* **191**: 381–392.
- Ilse D, Vaidya VG. 1956.** Spontaneous feeding response to colours in *Papilio demoleus* L. *Proceedings of the Indian Academy of Sciences* **43**: 23–31.
- Imhof L, Suárez M, Paganelli F, Facciuto G. 2013.** Interspecific hybridization among three species of

- Glandularia* (Verbenaceae) native to Argentina. *Acta Horticulturae* **1000**: 481–486.
- Johnson SD, Bond WJ. 1994.** Red flowers and butterfly pollination in the fynbos of South Africa. In: Arianoutsou-Faraggitaki M, Groves RH, eds. *Plant-animal interactions in Mediterranean-type ecosystems*. Dordrecht: Springer, 137–148.
- Kelber A, Pfaff M. 1999.** True colour vision in the orchard butterfly, *Papilio aegeus*. *Naturwissenschaften* **86**: 221–224.
- Kinoshita M, Shimada N, Arikawa K. 1999.** Colour vision of the foraging swallowtail butterfly *Papilio xuthus*. *Journal of Experimental Biology* **202**(2): 95–102.
- Kinoshita M, Stewart FJ, Ômura H. 2017.** Multisensory integration in Lepidoptera: insights into flower-visitor interactions. *BioEssays* **39**: 1600086.
- van der Kooij CJ, Pen I, Staal M, Stavenga DG, Elzenga JTM. 2016.** Competition for pollinators and intra-communal spectral dissimilarity of flowers. *Plant Biology* **18**: 56–62.
- Koshitaka H, Kinoshita M, Vorobyev M, Arikawa K. 2008.** Tetrachromacy in a butterfly that has eight varieties of spectral receptors. *Proceedings of the Royal Society B: Biological Sciences* **275**: 947–954.
- Kuznetsova A, Brockhoff PB, Christensen RHB. 2017.** lmer test package: tests in linear mixed effects models. *Journal of Statistical Software* **82**: 1–26.
- Lamas G. 2004.** Checklist: part 4A. Hesperioidea - Papilionoidea. In: Heppner JB, ed. *Atlas of Neotropical Lepidoptera*. Gainesville: Association for Tropical Lepidoptera Scientific Publishers, 1–428.
- Lewis AC, Lipani GA. 1990.** Learning and flower use in butterflies: hypotheses from honey bees. *Insect-Plant Interactions* **2**: 95–110.
- Lunau K, Maier EJ. 1995.** Innate colour preferences of flower visitors. *Journal of Comparative Physiology* **177**: 1–19.
- Maia R, Gruson H, Endler JA, White TE. 2019.** pavo 2: new tools for the spectral and spatial analysis of colour in R. *Methods in Ecology and Evolution* **10**: 1097–1107.
- May PG. 1985.** Nectar uptake rates and optimal nectar concentrations of two butterfly species. *Oecologia* **66**: 381–386.
- McCulloch KJ, Osorio D, Briscoe AD. 2016.** Sexual dimorphism in the compound eye of *Heliconius erato*: a nymphalid with at least five classes of photoreceptor. *Journal of Experimental Biology* **219**: 2377–2387.
- McCulloch KJ, Yuan F, Zhen Y, Aardema ML, Smith G, Llorente-Bousquets J, Andolfatto P, Briscoe AD. 2017.** Sexual dimorphism and retinal mosaic diversification following the evolution of a violet receptor in butterflies. *Molecular Biology and Evolution* **34**: 2271–2284.
- Niu Y, Chen Z, Stevens M, Sun H. 2017.** Divergence in cryptic leaf colour provides local camouflage in an alpine plant. *Proceedings of the Royal Society B: Biological Sciences* **284**: 20171654.
- Ômura H, Honda K. 2005.** Priority of color over scent during flower visitation by adult *Vanessa indica* butterflies. *Oecologia* **142**: 588–596.
- Osorio D, Vorobyev M. 2008.** A review of the evolution of animal colour vision and visual communication signals. *Vision Research* **48**: 2042–2051.
- R Core Team. 2019.** *R: a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.
- Rader R, Bartomeus I, Garibaldi LA, Garratt MP, Howlett BG, Winfree R, Cunningham SA, Mayfield MM, Anthony DA, Andersson GKS, Bommarco R, Brittain C, Carvalheiro LG, Chacoff NP, Entling MH, Foully B, Freitas BM, Gemmill-Herren B, Ghazoul J, Griffin SR, Gross CL, Herbertsson L, Herzog F, Hipólito J, Jaggar S, Jauker F, Klein AM, Kleijn D, Krishnan S, Lemos CQ, Lindström SAM, Mandelik Y, Monteiro VM, Nelson W, Nilsson L, Pattemore DE, Pereira N, Pisanty G, Potts SG, Reemer M, Rundlöf M, Sheffield CS, Scheper J, Schüepp C, Smith HG, Stanley DA, Stout JC, Szentgyörgyi H, Taki H, Vergara CH, Viana BF, Woyciechowski M. 2016.** Non-bee insects are important contributors to global crop pollination. *Proceedings of the National Academy of Sciences U.S.A.* **113**: 146–151.
- Ramos BCM, Trigo JR, Rodrigues D. 2019.** The specialization continuum: decision-making in butterflies with different diet requirements. *Behavioural Processes* **165**: 14–22.
- Renoult JP, Kelber A, Schaefer, HM. 2017.** Colour spaces in ecology and evolutionary biology. *Biological Reviews* **92**: 292–315.
- Rodrigues D, Goodner BW, Weiss MR. 2010.** Reversal learning and risk-averse foraging behavior in the monarch butterfly, *Danaus plexippus* (Lepidoptera: Nymphalidae). *Ethology* **116**: 270–280.
- Schaefer HM, Ruxton GD. 2011.** *Plant-animal communication*. Oxford: Oxford University Press.
- Scherer C, Kolb G. 1987.** Behavioral experiments on the visual processing of color stimuli in *Pieris brassicae* L. (Lepidoptera). *Journal of Comparative Physiology A* **160**: 645–656.
- Sison-Mangus MP, Briscoe AD, Zaccardi G, Knüttel H, Kelber A. 2008.** The lycaenid butterfly *Polyommatus icarus* uses a duplicated blue opsin to see green. *Journal of Experimental Biology* **211**: 361–369.
- Swihart CA. 1971.** Colour discrimination by the butterfly *Heliconius charitonius*. *Animal Behaviour* **19**: 156–164.
- Swihart CA, Swihart SL. 1970.** Colour selection and learned feeding preferences in the butterfly, *Heliconius charitonius*. *Animal Behaviour* **18**: 60–64.
- Vorobyev M, Osorio D. 1998.** Receptor noise as a determinant of colour thresholds. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **265**: 351–358.

- Weiss MR. 1991.** Floral colour changes as cues for pollinators. *Nature* **354**: 227–229.
- Weiss MR. 1995.** Associative colour learning in a nymphalid butterfly. *Ecological Entomology* **20**: 298–301.
- Weiss MR. 1997.** Innate colour preferences and flexible colour learning in the pipevine swallowtail. *Animal Behaviour* **53**: 1043–1052.
- Weiss MR, Papaj DR. 2003.** Colour learning in two behavioural contexts: how much can a butterfly keep in mind? *Animal Behaviour* **65**: 425–434.
- Wiemer AP. 2010.** *Biología de la polinización de especies de Asclepiadoideae (Apocynaceae) de la provincia de Córdoba.* Unpublished D.Phil. Thesis, Universidad Nacional de Córdoba with “*Biología de la polinización de especies de Asclepiadoideae (Apocynaceae) de la provincia de Córdoba.* Thesis, Universidad Nacional de Córdoba. Córdoba.
- Yuan F, Bernard GD, Le J, Briscoe AD. 2010.** Contrasting modes of evolution of the visual pigments in *Heliconius* butterflies. *Molecular Biology and Evolution* **27**: 2392–2405.
- Zaccardi G, Kelber A, Sison-Mangus MP, Briscoe AD. 2006.** Color discrimination in the red range with only one long-wavelength sensitive opsin. *Journal of Experimental Biology* **209**: 1944–1955.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Cumulative number of *A. vanillae* individuals that chose the rewarding colour vs. the number of training sessions.

**Figure S2.** Total number of observed flowers of *G. peruviana* (red) and *G. venturii* (lilac-purple) in the field to record visitation rate of *A. vanillae* butterflies during two subsequent flowering seasons (season I: November 2017 - March 2018 and season II: December 2018 - March 2019).

**Figure S3.** Normalized irradiance of illumination and reflectance spectra of background used in the calculation of the colour loci in the Maxwell triangle of a model butterfly.

**Table S1.** Average nectar production ( $\mu\text{L}$ ), sucrose concentration (% w/w) and sucrose amount (mg) in flowers of the focal *Glandularia* species ( $N = 10$  individuals).

**Appendix S1.** Equations used for calculations as implemented in the pavo v.2.2.0. package (Maia *et al.*, 2019) of the R program (R Core Team, 2019) and the Receptor Noise Model.

## SHARED DATA

Reflectance spectra are available at The Floral Reflectance Database (FReD; <http://www.reflectance.co.uk/>). *Glandularia peruviana* IDs #4325–4353, *G. venturii* IDs #4354–4382.