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ORIGINAL PAPER

Responses of butterflies to visual and olfactory signals of flowers of the bush lily *Clivia miniata*

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Abstract

Experimental studies of the use of visual and olfactory cues by flower-visiting animals can shed light on the evolution of floral signalling traits. We examined the functional significance of floral traits in *Clivia miniata* (Amaryllidaceae). This forest lily with large orange trumpet-shaped flowers is pollinated mainly by swallowtail butterflies and belongs to a lineage with ancestral bird pollination. We used *C. miniata* flowers varying in colour, orientation and scent, and arrays of artificial flowers varying in colour, pattern, orientation, size, shape, and scent to assess foraging preferences of the butterflies that pollinate *C. miniata*. Butterflies preferred orange over yellow colour morphs of *C. miniata* and preferred red and orange model flowers over yellow ones. Orange models with a central yellow target 'nectar guide' were favoured over plain orange models. Butterflies also favoured large over small model flowers and preferred to alight on upward-facing flowers. Addition of scent compounds emitted by *C. miniata* flowers increased butterfly visitation to model and natural flowers. These results identify the importance of particular combinations of visual and olfactory signals for attraction of swallowtail butterflies and shed light on the floral modifications associated with a shift from bird to butterfly pollination.

Keywords Advertising signals · Floral evolution · Floral traits · Lepidoptera · Perception · Pollinator behaviour · Pollinator cues

Introduction

Floral traits are commonly interpreted as an outcome of pollinator-mediated selection. This form of selection can arise from pollinator foraging choices that are influenced by perception, sensory bias and associative conditioning. Field-based studies of animal behavioural responses to visual and olfactory cues are therefore critical for understanding floral signal evolution (Schiestl and Johnson 2013). Although macroevolutionary studies have established broad correlations between floral traits and particular groups of flowers visitors (Van der Niet and Johnson 2012; Johnson and Wester 2017), experiments involving responses of animals to natural manipulated or artificial flowers are the most powerful way to determine the function of floral traits (Schemske and Bradshaw 1999; Campbell 2009). Studies of phenotypic selection involving standing trait variation (Sletvold and Ågren 2010; Sletvold et al. 2016; Caruso et al. 2019) and experimental evolution (Gervasi and Schiestl 2017) are also valuable, but in some cases, it is necessary by means of techniques such as hybridisation, to reintroduce phenotypic variation that may have been eliminated by selection (Schemske and Bradshaw 1999), or to use artificial or manipulated natural flowers to gain insights into floral function (Campbell 2009; Campbell et al. 2016; Policha et al. 2016). The advantage of using artificial flowers or manipulations of natural flowers is that this allows precise identification of traits that influence behaviour (Bell 1985; Mitchell-Olds and Shaw 1987; Fulton and Hodges 1999). Artificial flowers have proven particularly useful for identifying the relative efficacy of individual floral signals, and for determining preferential foraging cues used by pollinators (Ômura and Honda 2005; Goyret et al. 2007; Drewniak et al. 2020).

Most pollinators use overall colour as well as contrasting patterns as foraging cues (Briscoe and Chittka 2001; Chittka et al. 2001). Contrasting colour patterns can act as 'nectar

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guides' (Sprengel 1793; Johnson and Dafni 1998; Lunau et al. 2006) and are widespread throughout the angiosperms (Chittka et al. 1994; Weiss 1995). Floral scent often plays an important role in long-distance pollinator attraction (Metcalf and Metcalf 1992; Raguso 2008) and may reinforce foraging constancy (Dornhaus and Chittka 1999) as well as alter innate colour preferences (Yoshida et al. 2015).

Lepidoptera provide significant ecological and economic pollination services, yet remain comparatively poorly studied as pollinators (Rader et al. 2016). They have sophisticated sensory systems and colour is a primary cue used by foraging butterflies (for review see Kinoshita et al. 2017), with a number of species seemingly relying exclusively on colour while foraging (Andersson and Dobson 2003; Ômura and Honda 2005; Newman et al. 2012). Butterfly colour perception is some of the most developed of all animals and can range from ultraviolet to red (Silberglied 1984; Koshitaka et al. 2008). Nectar-seeking butterflies are typically attracted to blue (Lunau and Maier 1995), but some butterflies, particularly swallowtails, show preferences for longer wavelengths including yellow, orange or red (Crane 1955; Ilse and Vaidya 1956; Swihart and Swihart 1970; Scherer and Kolb 1987; Weiss 1997; Blackiston et al. 2011; Newman et al. 2012; Hirota et al. 2019). Butterflies exhibit strong innate colour preferences, but can also readily learn to associate food rewards with particular colours (Swihart and Swihart 1970; Goulson and Cory 1993; Weiss 1997).

A shift from bird to butterfly pollination occurred during the diversification of the genus *Clivia* and was associated with profound floral modifications (Kiepiel and Johnson 2014). *Clivia miniata* Lindl. Bosse occupies a derived position and is the only member of the genus with flowers that are upright, trumpet-shaped, and scented. The species is pollinated primarily by swallowtail butterflies (e.g. Figure 1a and c), while the other four species in the lineage have narrow unscented pendulous flowers and are all sunbird-pollinated (Kiepiel and Johnson 2014). The floral architecture of *C. miniata* facilitates wing pollination during brush and alight visits and provides a landing platform for foraging butterflies (Kiepiel and Johnson 2014). *Clivia miniata* flowers are usually orange with a central yellow target, but there is a rare morph (var. *citrina*) with uniform yellow floral coloration. *Clivia* represents an opportunity to examine the function of floral traits important for butterfly pollination in a clade where the ancestor can be reliably inferred to have typical pendant tubular bird-pollinated flowers.

The aim of this study was to identify the visual and olfactory cues used by butterflies that pollinate *C. miniata*. Given that the floral traits of *C. miniata* represent modifications for butterfly pollination, we hypothesised (1) that butterflies will

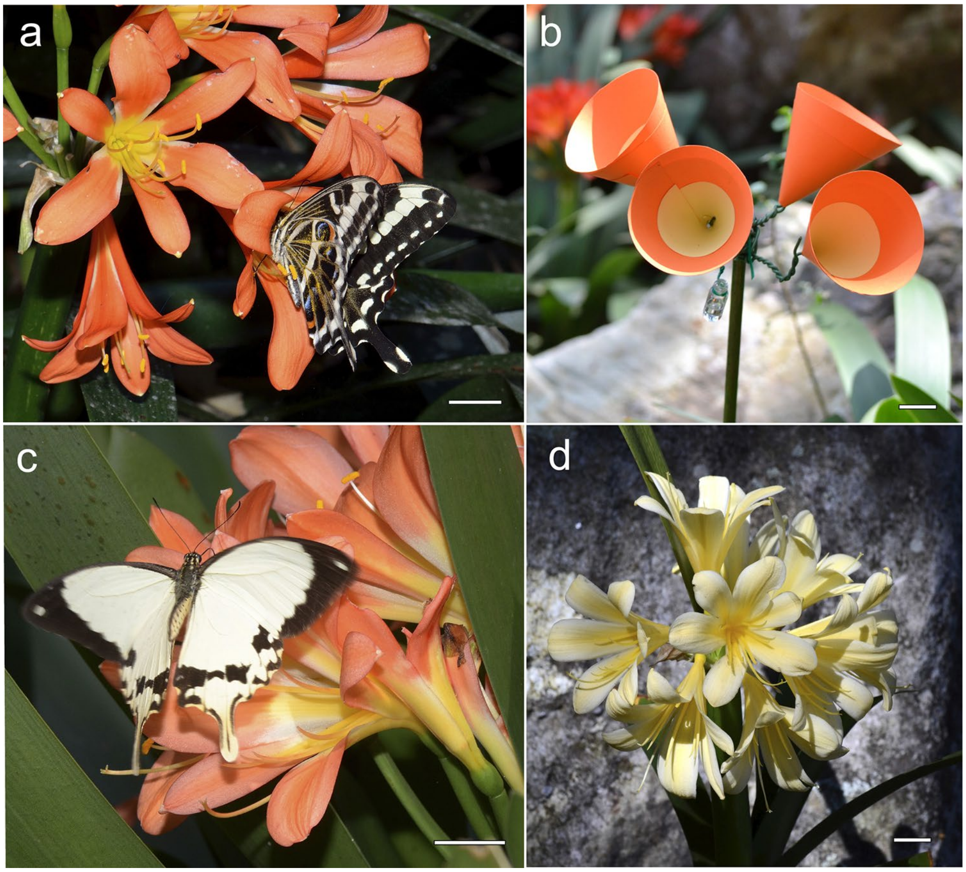


Fig. 1 **a** *Papilio ophidicephalus* (male) feeding on *C. miniata* at the MPNR site, exhibiting a large pollen load clustered around the ventral hind wing venation. **b** '*Clivia miniata*' paper model used in choice tests (here orange with yellow target), with vial containing artificial floral scent. **c** *Papilio dardanus cena* (male) exhibiting proboscis extension reflex during a typical brush visit to *C. miniata* at the UNR site. **d** Emasculated *C. miniata* var. *citrina* inflorescence used in paired-choice tests at the UNR site. Scale bars = 20 mm

favour orange flowers with a central yellow target pattern over other colours and patterns, (2) that butterflies will be more attracted to, and more likely to settle on, flowers which face upwards, and (3) that butterflies would prefer scented over unscented flowers.

Methods

Study sites

Experiments were performed in the forest habitats of *Clivia miniata* during the Austral spring (flowering season August to October) from 2010 to 2017, at two sites in KwaZulu-Natal Province, South Africa. One site was inland (approximately 90 km from the coast), located in the Mistbelt forests of the Karkloof Midlands (Mbona Private Nature Reserve, 29° 17′ S; 30° 21′ E, ca, 1300 m a.s.l., hereafter referred to as MPNR). The other was a coastal site, situated in coastal scarp forest (Umtamvuna Nature Reserve, 31° 00′ S, 30° 09′ E, ca. 150 m a.s.l., hereafter referred to as UNR). These two sites are located approximately 185 km apart.

Spectral assessment and model design

Floral spectral reflectance measurements (Fig. 2a) of the corolla of *C. miniata* were taken from flowers at MPNR (mean of 12 flowers from 12 plants) and UNR (mean of 10 flowers from 10 plants) sites. Spectra of flowers of *Clivia miniata* var. *citrina* (mean of 10 flowers from 10 plants) were taken from nursery raised plants (Cycad Centre, KwaZulu-Natal, RSA), as this form is extremely rare in the wild. Spectral reflectance (300–700 nm) was measured using an Ocean Optics S2000 spectrophotometer (Ocean Optics, FL, USA) coupled with a fibre optic reflectance probe (QR-400-7-UV–VIS; 400 µm, Johnson and Andersson 2002).

Paper model flowers were constructed to match the shape and size of *C. miniata* and *C. gardenii* flowers. Paper was chosen that most closely approximated the colour of *C. miniata* or co-flowering species (see discussion). "Brilliant Bright Board" was used for blue, green, orange and red colours, while "Marlin" paper was used for pink and pastel yellow colours (Fig. 2b). Pink and pastel yellow papers of a different brand were chosen for their closer approximation to pink flowered species and *C. miniata*, respectively. Both pink and yellow paper possessed a higher UV component than that of the other model colours, consistent with UV reflectance of the pastel yellow portions of *C. miniata* flowers being higher than the orange portions (Fig. 2a).

Clivia miniata models (Fig. 1b) consisted of cones 74 mm in depth and 73 mm in width, thus matching the size of actual flowers (see Kiepiel and Johnson 2014). Reduced size models were 1/3 of this size. *Clivia gardenii* models were tubular with a length of 40 mm and an entrance width of 10 mm (see Kiepiel and Johnson 2014).

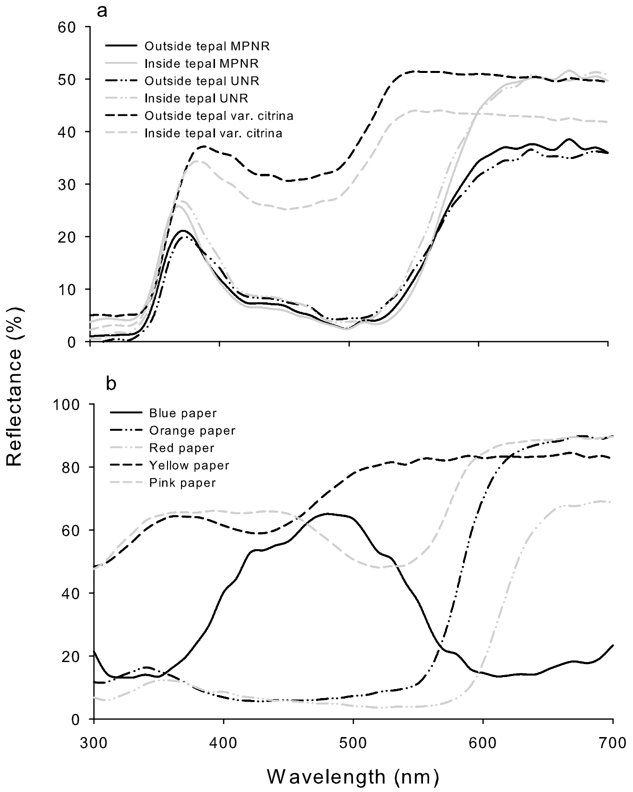


Fig. 2 **a** Spectral reflectance of wild *C. miniata* (two sites) and commercially raised *C. miniata* var. *citrina* flowers. **b** Spectral reflectance of model paper used in artificial flower arrays. Curves illustrate mean reflectance for real and model flowers, where *n* represents the number of samples, each taken from a separate plant or sheet of paper, respectively. *Clivia miniata* (MPNR; $n=12$, UNR; $n=10$), *C. miniata* var. *citrina* ($n=10$), model paper ($n=6$, for all six colours)

Artificial pedicels were constructed from green wire and were attached to 'peduncles' manufactured from 1 m long wooden dowel rods (8 mm diameter), which were painted green (Spectra Spray, lead free aerosol spray paint, Brilliant Green). Four 'pedicels' were placed onto a 'peduncle' to create each artificial inflorescence (Fig. 1b). Model flowers were orientated upwards at an angle of 45° (approximating *C. miniata*) or downwards (approximating *C. gardenii*).

Model flower arrays

Choice experiments began in the morning, prior to the commencement of butterfly activity (06h00), and trials were terminated when butterfly activity had ceased (c. 16h00). Butterflies typically became active around 08h00 on sunny days and were inactive during overcast or rainy weather (below temperatures circa 22 °C). Our preliminary behavioural experiments using wild caught butterflies housed in nylon walk-in cages (2 × 2 × 2 m) indicated that although the animals visited model flower arrays in captivity (with and without centrally placed Eppendorf® tubes containing 20% sucrose solutions), the wings of the butterflies frayed upon contact with the mesh and the butterflies quickly deteriorated. We thus opted to use field-based experiments which also have the advantage that they better reflect the foraging preferences of wild butterflies that account for selection on floral traits. Arrays were positioned in the forest in flowering *C. miniata* populations and consisted of a choice between two or more natural or artificial inflorescences. These inflorescences were placed approximately 100 cm apart, and model peduncles (dowel rods) were pushed into the soil or wedged between rocks so that model flowers approximated the height of surrounding real inflorescences and were at least 5 m (but no more than 10 m) from natural *C. miniata* flowers allowing the recording of a definite unmistakable behavioural choice for approach. We did not record proboscis extension reflex as this behaviour was difficult to observe in butterflies in flight. Preliminary experiments with field caught butterflies indicated no proboscis extension responses when butterflies were held close to either colour paper discs (with or without artificial scent) or real flowers (with or without artificial scent). The location of model inflorescences were randomised every 10 min in order to avoid any potential position effects (e.g. butterfly learning, sunspots etc.). The time of day of each visit and species was recorded. The following behaviours were recorded for model and natural inflorescences: approaches within 15 cm of an ‘inflorescence’; brushes, when the butterflies did not settle and brushed the models with wings or legs (Fig. 1c shows this behaviour on an actual flower); and alights, when butterflies landed on the models. Visit duration, probing behaviour and probing time were also recorded.

To test responses of *Papilio dardanus cena*, the dominant visitor at UNR, to various colours, we used an array of *C. miniata*-shaped models consisting of five plain (unpatterned) colour choices; pink, blue, yellow, orange, and red (see supplementary Table S1 for all site details and experimental duration).

To test responses of the overall butterfly assemblage to colour patterns at MPNR, we used a model array comprising of six *C. miniata*-shaped choices (three plain colours and three with targets); orange, red, yellow, orange with yellow target, red with yellow target, and yellow with red target models. Data for this experiment were pooled due to generally low sample sizes, and we assumed that overall preferences of the butterfly assemblage determine floral trait selection (assuming equal effectiveness between species). We also tested plain orange models versus orange ones with a yellow target at a different site.

To test the effects of flower size, we used a model array consisting of plain orange *C. miniata*-shaped models that were either full size or reduced to one-third size. We also compared plain orange models matching the size and shape of *C. miniata* to those matching the size and shape of *C. gardenii*.

To test the role of scent we used a model array consisting of orange with yellow target *C. miniata*-shaped models, either with 1000 µl of an artificial *C. miniata* scent blend (housed in a small vial in the centre of the ‘inflorescence’) or a paraffin oil control (Fig. 1b). This scent blend was a mixture of equal proportions of Benzyl Alcohol (Sigma-Aldrich), Benzaldehyde (Sigma-Aldrich), and Benzyl benzoate (Sigma-Aldrich)—5 µl of each compound into 985 µl unscented paraffin liquid. These three compounds were chosen for their ubiquitous presence in *C. miniata* scent (Kiepiel and Johnson 2014). Scent vials were shaken every 10 min and replaced every hour, and scented models were kept separate to unscented models to prevent scent contamination.

Arrays with natural flowers

Plants were kept in an insect-proof nylon mesh cage and flowers emasculated prior to anthesis to avoid genetic contamination of wild populations. To assess whether butterflies prefer the common orange morph of *C. miniata* over the rarer yellow morph (var. *citrina*, Fig. 1d), we compared rates of butterfly visitation to the two morphs using a paired design. We used inflorescences with eight open flowers and replaced them with fresh ones daily. Inflorescences were attached to the same green wire used to manufacture model arrays and positioned and randomised as for model flower arrays above.

To test the effect of floral orientation, we presented *C. miniata* flowers in their usual upright orientation versus *C. miniata* flowers manipulated into a pendulous orientation. We used four *C. miniata* flowers per inflorescence (emasculated virgin flowers were cut at the base of the pedicel), which were attached to artificial pedicels and secured to model peduncles in a similar fashion to artificial arrays (see Fig. S4).

To test the effect of scent supplementation, vials containing paraffin with scent compounds described above were hung from pedicels of scent-enhanced inflorescences while vials containing pure paraffin were hung from pedicels of control inflorescences (arrays secured as with colour morph bioassay above).

Statistical analysis

Data were analysed using generalised linear models implemented in SPSS Version 25 (IBM Corp.). Each hour-long observation period was used as a subject in generalised estimating equations (GEEs) to control for any potential lack of independence among the observations in that period. Counts of butterfly behavioural events were modelled with a negative binomial distribution with a log link function, while the proportions of butterflies choosing a particular model or real flowers was modelled using a binomial distribution with a logit link function. First-choice proportions were considered significant if 95% confidence intervals did not overlap the 0.5 proportion value of equal choice among two options. GEE models incorporated an exchangeable correlation matrix and significance was tested using Wald Statistics. We used the sequential-Šidák method for post hoc comparisons among means. For graphical presentation of marginal means in the original scale measurement, log or logit data were back-transformed, yielding asymmetrical standard errors.

Results

Model flower arrays

Papilio dardanus cena dominated the visitor assemblage at UNR and showed significant discrimination among colours (Wald $\chi^2 = 21.050$, $P < 0.001$) with pink approached least and orange the most often (Fig. 3). Few butterflies brushed or alighted on these models (Table S1).

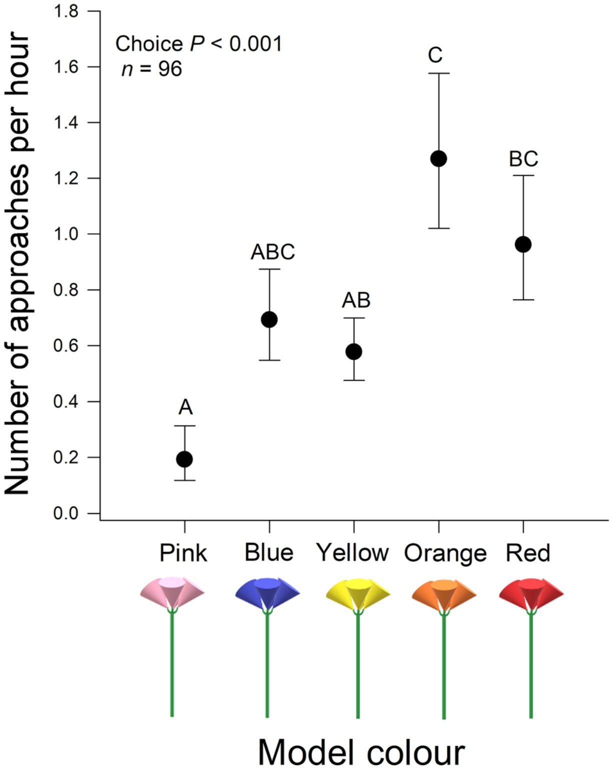


Fig. 3 Behavioural responses of *Papilio dardanus cena* to model arrays consisting of five plain (unpatterned) colour choices. Bioassays conducted at the UNR site in the flowering season of 2013. Observations ($n = 120$) recorded over 5 full days, and over 26 unique time blocks. Approaches; $n = 96$ (brushes; $n = 16$, alights; $n = 8$)

Models in the colour pattern choice array at MPNR were visited by five species of butterflies (*Nepheronia argia, P. dardanus cena, Papilio echerioides echerioides, Papilio euphranor, Papilio ophidicephalus*), the data for which were pooled in analyses as the sample size for each species was too low to assess differences among species. Model colour significantly influenced approaches (Wald $\chi^2 = 39.020$, $P < 0.001$; Fig. 4a) and alights (Wald $\chi^2 = 8.056$, $P = 0.018$) by this butterfly assemblage, but had no impact on the number of brush visits (Wald $\chi^2 = 4.078$, $P = 0.130$). Although targets alone did not significantly affect any butterfly foraging behaviour (Table S1), there was a significant interaction between colour and target in the number of approaches to the colour pattern array (Wald $\chi^2 = 8.235$, $P = 0.016$). With the exception of three *P. nireus lyaeus* individuals, models in the binary colour pattern choice array at the UNR site were visited exclusively by *P. dardanus cena* and we therefore analysed only data for the latter species. The model with a central yellow target was strongly preferred by *P. dardanus cena* (Fig. 4b) and was approached (Wald $\chi^2 = 24.592$, $P < 0.001$) and brushed (Wald $\chi^2 = 6.443$, $P = 0.011$) significantly more often. There were no alights on these models. Almost 75% of butterfly approaches were to models with targets (Fig. 4b, Table S1 first choice).

The array at MPNR testing the effects of flower size was visited by six butterfly species, but we opted not to pool this data for the GEE models and analysed data from the principal visitor *P. ophidicephalus* (Table S1). *Papilio ophidicephalus* which significantly preferred larger models in terms of approaches per hour (Wald $\chi^2 = 6.863$, $P = 0.009$; Fig. S1). Over 93% of butterflies approached the larger models as their first choice compared to the smaller ones (Table S1). Very few butterflies brushed or alighted on these models (Table S1).

Several butterfly species visited the array of model flowers shaped either like *C. miniata* or *C. gardenii,* the data for which were pooled for analyses as the sample size for each species was too low to assess differences among species (Fig. S2 and Table S1). We found a strong significant trend for approaching butterflies to favour models representing *C. miniata* (Wald $\chi^2 = 9.842$, $P = 0.002$; Fig. S2), with almost 95% of butterflies approaching *C. miniata* models as a first choice (Table S1).

In the experiments testing the addition of scent to model flowers (Fig. S3), over 65% of butterflies at MPNR and almost 60% of butterflies at UNR approached scented models (Table S1 first choice). There was a significant overall preference for scented models in terms of approaches (Wald $\chi^2 = 19.597$, $P < 0.001$) and brushes (Wald $\chi^2 = 12.739$, $P < 0.001$) at the MPNR site (Fig. S3a and c), and approach and alight visits made by *N. argia* to scent-supplemented models were significantly more frequent than those to

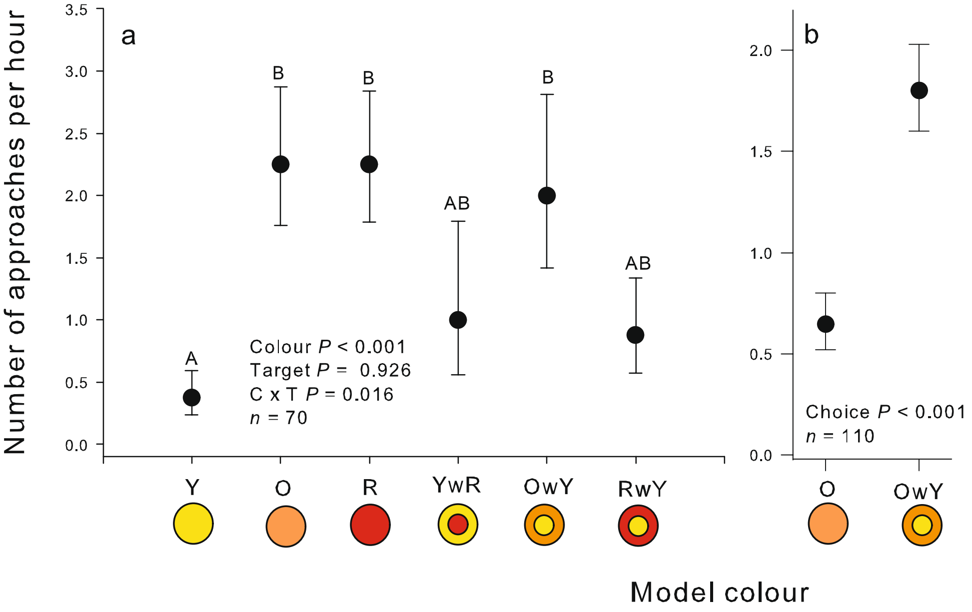


Fig. 4 **a** Behavioural responses of five butterfly species (*Nepheronia argia, Papilio dardanus cena, P. echerioides echerioides, P. euphranor*, and *P. ophidicephalus*) to model flower arrays consisting of six *C. miniata*-shapes choices (three plain colours and three with targets). Colours from left to right are as follows: yellow, orange, red, yellow with red target, orange with yellow target and red with yellow target. Bioassays conducted at the MPNR site in October 2010. Observations ($n = 121$) recorded over 2 full days, and over 8 unique time blocks. Approaches; $n = 70$ (brushes; $n = 26$, alights; $n = 25$). **b** Behavioural responses of *P. dardanus cena* to two *C. miniata*-shaped model flower arrays; plain orange versus orange with a yellow target. Bioassays conducted at the UNR site in the flowering season of 2013. Observations ($n = 123$) recorded over 5 full days, and over 45 unique time blocks. Approaches; $n = 110$ (brushes; $n = 13$, alights; $n = 0$)

unscented ones (Fig. S3a and e). At the UNR site, there was a significant overall preference for scented models in terms of approaches (Wald $\chi^2 = 7.883$, $P = 0.005$), and approach and brush visits made by *P. dardanus cena* to scent-supplemented models were significantly more frequent than those to unscented models (Fig. S3b and d).

Arrays with natural flowers

In binary choices between var. *miniata* and var. *citrina* in the 2013 season, 68% of butterflies first approached var. *miniata*, while in the 2014 season, approximately 77% of butterflies first approached var. *miniata* flowers (Table S1, first choice). In both the 2013 and 2014 seasons, var. *miniata* flowers were approached, brushed, and alighted upon a significantly greater number of times per hour by *P. dardanus cena* than were var. *citrina* (Fig. 5). *Papilio nireus lyaeus* and the pierid *Belenois zochalia zochalia* also approached var. *miniata* inflorescences significantly more often than on those of var. *citrina* in the 2014 season (Fig. 5b). There were no significant interactions between butterfly species and choice of colour variety in the 2014 season (Fig. 5b, d and f).

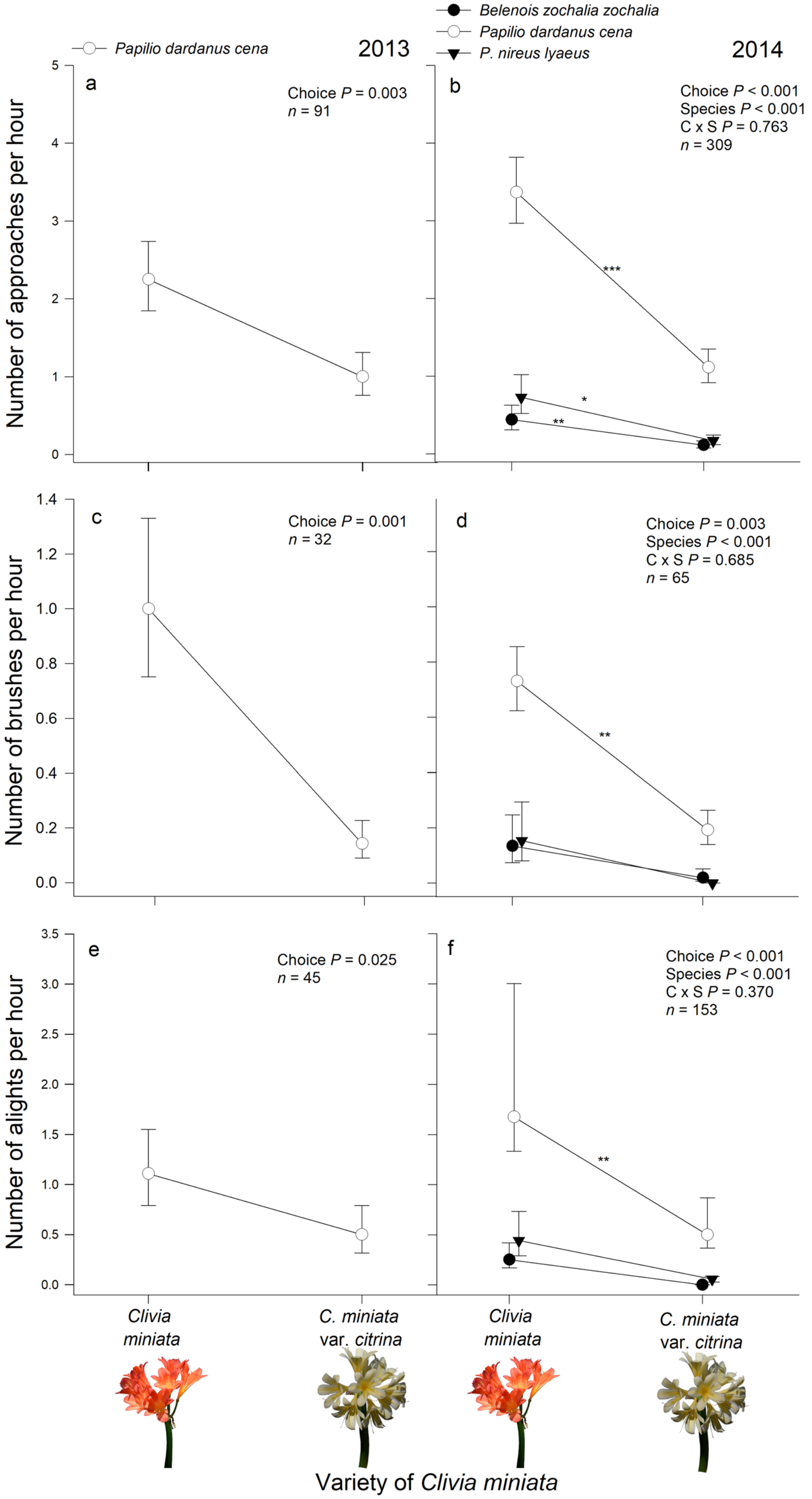
There was no overall difference in first choice of approach by butterflies in relation to flower orientation (Table S1, first choice). There was a significant overall preference for upright flowers in terms of brushes (Wald $\chi^2 = 7.060$, $P = 0.008$) and alights (Wald $\chi^2 = 5.067$, $P = 0.024$) compared to pendulous orientations (Fig. S4b and c), and *P. dardanus cena* individuals alighted significantly more often on the upright-oriented flowers (Fig. S4c).

A significantly higher proportion of butterflies chose the scent-supplemented natural inflorescences (Table S1, first choice). Individuals of *P. dardanus cena* approached scent-supplemented flowers significantly more frequently than they did the control flowers (Wald $\chi^2 = 7.883$, $P = 0.005$), but there were no significant difference found in the number of brush visits (Wald $\chi^2 = 2.379$, $P = 0.123$) or alighting's (Wald $\chi^2 = 0.574$ $P = 0.448$) with the addition of scent (Fig. 6).

Discussion

Our results corroborate those of previous studies demonstrating that butterflies are highly sensitive to overall colour as well as colour pattern combinations (Goulson and Cory 1993; Kinoshita et al. 1999, 2017; Ômura and Honda 2005; Koshitaka et al. 2011). Results from this study suggest that flower colour is a key advertising signal used by *C. miniata* for attraction of the diverse assemblage of butterflies which pollinate this lily. Butterflies frequently exhibited behavioural responses involving directional flight pattern

Fig. 5 Behavioural responses of butterfly species to two real flower choices consisting of *C. miniata* versus *C. miniata* var. *citrina* inflorescences at the UNR site over two consecutive seasons (2013 and 2014). 2013 observations ($n = 168$) recorded over 3 full days, and over 28 unique time blocks. Approaches; $n = 91$, brushes; $n = 32$, alights; $n = 45$. 2014 observations ($n = 527$) recorded over 5 full days, and over 52 unique time blocks. Approaches; $n = 309$; brushes; $n = 65$, alights; $n = 153$



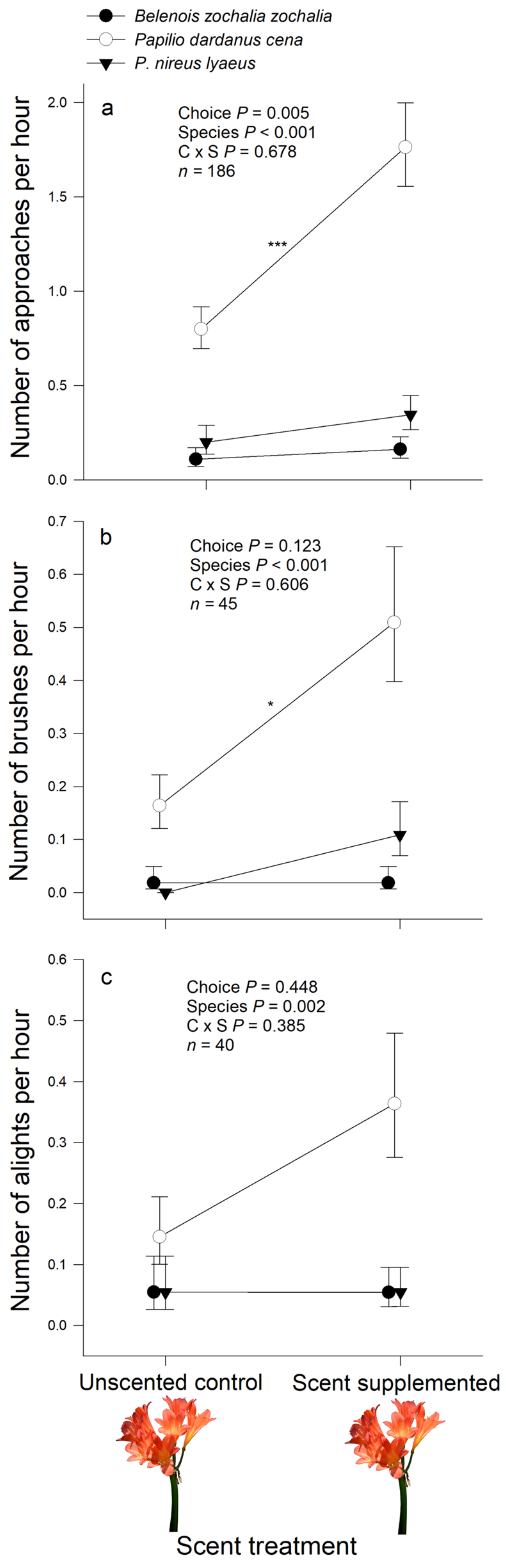


Fig. 6 Behavioural responses of three butterfly species (*Belenois zochalia zochalia*, *Papilio dardanus cena*, and *P. nireus lyaeus*) to two real flower arrays consisting of *C. miniata* flowers; unscented (paraffin control) versus scent supplemented. Bioassays conducted at the UNR site in the flowering season of 2014. Observations ($n = 272$) recorded over 5 full days, and over 25 unique time blocks. Approaches; $n = 186$, brushes; $n = 46$, alights; $n = 40$

adjustment towards the target stimulus from distances of up to 15 m. Bioassays indicated that orange and red models were preferred over yellow and pink, but not over blue. A preference for blue colours is very common in food-seeking insects including butterflies (Lunau and Maier 1995; Kelber 1997; Kelber et al. 2003). A number of butterflies show primary colour preferences for blue and secondary preferences for orange and red (see Kinoshita et al. 2017). In this study, *P. dardanus cena* which is the primary pollinator of *C. miniata* in coastal forests (Kiepiel and Johnson 2014), showed a strong preference for orange over yellow models (Fig. 3) and also preferred the orange-flowered var. *miniata* over the yellow-flowered *C. miniata* var. *citrina* (Fig. 5). In general, when offered a choice, butterflies strongly preferred the orange flowers of *C. miniata* var. *miniata* over the yellow flowers of *C. miniata* var. *citrina* (Table S1). This helps to explain why var. *citrina* remains rare in wild populations.

In the classical paradigm of floral syndromes, butterfly pollination is associated with vivid floral colouration including red (Faegri and van der Pijl 1979). Preferential foraging on flowers with red and orange colours has previously been demonstrated in South Africa for the nymphalid *Aeropetes tulbaghia* (Johnson 1994; Johnson and Bond 1994; Newman et al. 2012) and similar preferences have been documented for other butterflies in the region (Butler and Johnson 2020; Daniels et al. 2020). It is well established that most butterflies can associate colour with nectar rewards and thus show lability in colour preferences (Goulson and Cory 1993; Weiss 1997; Kinoshita et al. 1999; Drewniak et al. 2020). Swallowtails demonstrate variable innate colour preferences; some, such as *Papilio demoleus,* prefer blue and purple (Ilse and Vaidya 1956), while others, such as *Papilio xuthus,* favour yellow and red (Kinoshita et al. 1999). The spicebush swallowtail *Papilio troilius* demonstrates spontaneous feeding preferences for blue and to a lesser extent orange, with responsiveness to orange increasing with behavioural conditioning (Swihart 1970). Similar preferences have also been found in nymphalids and *Heliconis charitonius* displays spontaneous preferences clustered around blue and orange/red (Swihart and Swihart 1970), while *Danaus plexipus* shows strong innate preferences for orange and yellow (Blackiston et al. 2011). Field caught swallowtails including *P. dardanus cena*, *P. echerioides echerioides, P. nireus lyaeus*, and *P. ophidicephalus* readily fed on both blue and orange model flowers containing artificial sucrose rewards (I, Kiepiel, unpublished data) and these butterflies were observed visiting blue-flowered forest margin and grassland plant species on numerous occasions (I, Kiepiel, personal observation). Blue, red, and orange thus appear to be the colours that are most often preferred across a range of butterfly species.

The response of butterflies to various target patterns differed between sites (Fig. 4), with a strong preference for a yellow target on an orange background being evident at the site dominated by the swallowtail *P. dardanus cena* (Fig. 4b). A variance in preferences for colour targets across different sites visited by discrete butterfly assemblages (Fig. 4) suggests that preference for colour contrasting patterns may not be a universal across all butterfly species that pollinate *C. miniata*. Using artificial paper flowers, nectar guides comprising of a simple coloured spot have been shown to stimulate proboscis extension in the cabbage white butterfly *Pieris rapae*, improving both efficiency and consistency of foraging (Kandori and Ohsaki 1998). Other studies of papilionids suggest that *P. xuthus* uses target-background intensity contrast for landing (Koshitaka et al. 2011) as well as bullseye patterns (Hirota et al. 2019), while the southern birdwing *Troides minos* uses the white coloured UV absorbing bract of *Mussaenda frondosa* L. as an important long-distance signalling cue (Borges et al. 2003). Insect preference for contrasting colour patterns, including “nectar guides”, particularly at close range distances is well established (Waser and Price 1985; Kandori and Ohsaki 1998; Dafni and Giurfa 1999; Hansen et al. 2012). It is possible that the preference of *P. dardanus cena* for target patterns was learned through association with local *C. miniata* plants, but further experiments would be required to test if this is an innate preference. It is also possible that orange with yellow target models were preferred over other targeted models simply because of the achromatic effect of this combination being brighter overall than other colour combinations.

This study demonstrated that the emperor swallowtail *P. ophidicephalus* strongly preferred larger over smaller models (Fig. S1), supporting earlier work showing preferences for larger size models by *P. demoleus* (Vaidya 1969). Floral size and architecture are typically governed by requirements for pollinator attraction (Faegri and van der Pijl 1979; Armbruster 1996) and both floral and inflorescence size influence pollinator visitation (Bell 1985). Preferences for larger flowers and floral displays have been described for many species of Lepidoptera (Vaughton and Ramsey 1998; Arroyo et al. 2007; Pohl et al. 2011).

Papilio ophidicephalus together with four other swallowtails and the pierid *N. argia* strongly favoured models resembling *C. miniata* over those resembling the pendulous flowered *C. gardenii* (Fig. S2, Table S1 first choice). However, this may simply reflect differences in size between the models, as the orientation of actual *C. miniata* flowers did not influence the number of approaches and influenced only alighting behaviour (Fig. S4, Table S1). Butterflies were unable to enter the corolla chamber in downwards orientated *C. miniata* flowers and feeding was completely prevented. Experimental manipulation of floral orientation in *Geranium refractum* Edgeworth & Hooker from a downward- to an upward-facing orientation has been found to shift pollination from specialisation (i.e. bumblebees) to generalisation, with an associated reduction in pollen transfer efficiency (Wang et al. 2014). In *Zaluzianskya* F.W. Schmidt, floral orientation has been shown to be critical for hawkmoth pollination, providing compelling evidence for the role of floral orientation in pollinator shifts and reproductive isolation (Campbell et al. 2016). Manipulation of *Aquilegia pubescens* Coville flowers into a pendent orientation reduced hawkmoth visitation by tenfold (Fulton and Hodges 1999). Experimental manipulation of *Nicotiana attenuata* Steud. flowers from an upright (i.e. 45° angle) to that of a downward-facing orientation (i.e. − 45° angle) resulted in a significant reduction in pollen delivery, virtually negated pollen removal, and resulted in a significantly lower foraging success for *Manduca sexta* hawkmoths (Haverkamp et al. 2019).

Butterflies readily approached flower models based on colour alone, but other visual or olfactory floral traits are clearly required to stimulate the level of brush and alighting behaviour that was observed for real flowers. Visually such traits may include the outline of the corolla and tepals or the tactile feel of the flower including the protruding stamens and style. The visual preferences of butterflies are diverse, and complex interactions exist between various sensory modalities such that synergy between advertising signals can result in behavioural shifts that differ from responses to individual floral cues alone (Kinoshita et al. 2017; Balamurali et al. 2020; Franzke et al. 2020). Although the role of scent signals in butterfly pollination systems requires clarification (Andersson et al. 2002), it is likely that scent plays a role in enhancing attractiveness of flowers to some, and perhaps even the majority of, butterfly species. In this study, *P. dardanus cena* brushed scented models significantly more than unscented models and scent supplementation increased the number of approach and brush visits to *C. miniata* flowers (Fig. 6a and b). Although swallowtail butterflies are known mainly for their responses to flower colour, other studies have shown that scent can modify colour preferences (Yoshida et al. 2015). Experimental addition of scent to less favoured colour targets has also been shown to increase their attractiveness to the nymphalid *Vanessa indica* but did not affect visitation to favoured yellow targets (Ômura and Honda 2005).

Conclusions and perspectives

Colour plays a primary role as an advertising signal in *C. miniata*. The overall preference of butterflies for orange and red colours may explain the natural rarity of yellow-flowered forms of *C. miniata*. This rarity may also be reinforced by selection for contrasting colour patterns and our initial hypothesis that butterflies will favour orange flowers with a central yellow target pattern over those with other colours and patterns was generally supported (Fig. 4). Our second hypothesis that butterflies will be more attracted to, and more likely to settle on, flowers which face upwards was supported only in terms of alighting behaviour (Fig. S4), reflecting butterfly foraging lability and the role of orientation as a floral isolating mechanism. Our third hypothesis that butterflies prefer scented over unscented flowers was also generally supported (Figs. 6, S3), consistent with switch from unscented to scented flowers during the transition from bird to butterfly pollination in *Clivia* (Kiepiel and Johnson 2014). The shift from bird to butterfly pollination in *Clivia* was also associated with the evolution of upward-facing trumpet-shaped flowers with a yellow target (Kiepiel and Johnson 2014) and the present study provides a demonstration of the functional significance of these trait modifications.

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Availability of data Data can be made available upon article acceptance.

Declarations

Conflict of interest All authors declare that they have no conflicts of interest.

Consent for publication All authors consent to publication.

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