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ORIGINAL PAPER

Colour preferences of *Tetragonula carbonaria* Sm. stingless bees for colour morphs of the Australian native orchid *Caladenia carnea*

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Abstract
Innate colour preferences promote the capacity of pollinators to find flowers, although currently there is a paucity of data on how preferences apply to real flowers. The Australian sugarbag bee (*Tetragonula carbonaria* Sm.) has innate preferences for colours, including UV-absorbing white. Sugarbag bees are pollinators of the terrestrial orchid *Caladenia carnea* R.Br., which has both white and pink morphs. In laboratory conditions, we tested flower-naïve bees with the white and pink flower morphs revealing a significant preference for the white morph, consistent with experiments using artificial stimuli. In experiments to understand how bees may select food-deceptive orchids following habituation to a particular colour morph, we observed a significant increase in choices towards novel white flowers. We also observed that the presence of a UV-reflecting dorsal sepal signal significantly increased bee choices compared to flowers that had the UV signal blocked. Our findings demonstrate that innate preference testing of insect pollinators with artificial stimuli is replicated in a biologically significant scenario with flowers. The findings also underscore how food-deceptive orchids can receive sufficient pollinator visits to ensure pollination by having different morphs that draw on the innate preferences of bees and their ability to make decisions in a complex ecological setting.

Keywords Flower · Orchids · Pollination · Innate preferences · Ultraviolet

Introduction

Plant–pollinator interactions provide important insights into how complex biological partnerships exist (Barth 1985; Sargent and Ackerly 2008; Mitchell et al. 2009). Bees, in particular, are an important model in neuroethology for understanding how the building blocks of sensory perception (Frisch 1914; Srinivasan and Lehrer 1988; Dyer et al. 2011; Reser et al. 2012; Hempel de Ibarra et al. 2014) influence how flowers visited by bees may have evolved specific signals (Chittka and Menzel 1992). Darwin (1877) postulated that innate preferences could allow flower visitors to more easily find flowers, and testing using artificial stimuli in several species of bees has revealed innate colour preferences that could act to influence bee choices (Giurfa et al. 1995; Gumbert 2000; Rohde et al. 2013; Dyer et al. 2016a). However, there is currently a paucity of data on how colour preferences observed in bees for artificial stimuli might represent the actual choices for real flowers. Understanding these principles is important for building a better understanding of how sensory perception of bee pollinators may shape plant communities.

Colour perception requires the presence of multiple photoreceptors, and bees have been shown to have a phylogenetically conserved trichromatic visual system based on ultraviolet-, blue- and green-sensitive photoreceptors (Briscoe and Chittka 2001). In this respect, flowers in both the Northern (Chittka and Menzel 1992; Arnold et al. 2009; Shrestha et al. 2014) and Southern Hemispheres (Dyer et al. 2012;

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Bischoff et al. 2013; Shrestha et al. 2013) are typically characterised by spectral signatures with marker points at about 400 and 500 nm, which closely match the optimal colour processing of bee trichromats (Helversen 1972; Chittka and Menzel 1992). However, there is strong evidence that the colour vision of different pollinators significantly influences what colour flowers evolve. For instance, in South America, both white and red hummingbird-pollinated flowers differ from bee-pollinated flowers in their reflection properties for ultraviolet (UV) light (Lunau et al. 2011). Specifically, red flowers lack UV signals and, thus, the predominately long wavelength signals do not modulate the trichromatic vision of bees, whereas hummingbird-pollinated white flowers do reflect UV, which results in achromatic stimuli that are difficult to detect since bees do not process brightness differences (Kevan et al. 1996; Spaethe et al. 2001; Dyer et al. 2007; Lunau et al. 2011; Ng et al. 2018). For common poppy (*Papaver rhoeas*) flowers, it was found that in the Middle East where it is pollinated by a red-sensitive beetle the flowers reflect only red light, but in Europe, where it is pollinated by bees, the flowers also reflect ultraviolet light (van der Kooi and Stavenga 2019). Thus, UV signalling from broadband natural colours can be complex and depend on which wavelengths of UV are reflected, and it is important to test different animals to understand how the building blocks of perception may take effect in biological systems (Kemp et al. 2015).

Recently, there has been increased interest in stingless bees and how these insects interact with their environment in a way that enables successful pollination (Hrncir et al. 2016). The sugarbag bee (*Tetragonula carbonaria* Sm.), is a small (1.13 ± 0.02 mm intertegulae span; mean ± SD; Dyer et al. 2016a, b) native Australian bee that lives in colonies of about 5000 individuals (Heard 2016). The sugarbag bee is an important pollinator of both native, and some agricultural, plant species (Heard 1999, 2016), and is amenable to experimental lab testing conditions (Norgate et al. 2010; Spaethe et al. 2014). The colour preferences of this species have been measured with broadband colour stimuli, revealing a significant preference for the blue and blue-green regions of hexagon colour space. In particular, a UV-absorbing white colour card strongly stimulates bee colour opponent vision and was the most preferred colour stimulus (Yang et al. 2004; Dyer et al. 2016a). Interestingly, UV-absorbing white flowers are frequently observed in surveys of bee-pollinated species (Kevan et al. 1996; Dyer et al. 2012; Bischoff et al. 2013). However, colour is a complex stimulus mediated by multiple factors (Kemp et al. 2015; van der Kooi et al. 2019), and in the sugarbag bee, green contrast was also a main factor implicated in mediating bee choices (Dyer et al. 2016a).

Orchids (Family Orchidaceae) employ an extraordinary range of reproductive strategies to achieve pollination, including the use of deceit to exploit the sensory systems and perceptual biases of Hymenopteran pollinators (Wong and Schiestl 2002; Peakall 2007; Phillips et al. 2009; Gaskett 2011). In this regard, the role of colour signals in orchids that use food mimicry to achieve pollination remains poorly understood (Jersáková et al. 2012, 2016). Bees, including the sugarbag bee, are known pollinators of the terrestrial orchid *Caladenia carnea* (Adams et al. 1992; Kuiter 2016), a widespread species endemic to south-eastern Australia. *Caladenia carnea* is believed to be a food-deceptive orchid that achieves pollination through colour mimicry of food-rewarding flowers. Interestingly, the species is also colour polymorphic, with both white and pink forms of the flower present in the same environment (Jones 2006 and Pers. obs authors). Both the orchid's pollination syndrome and its striking colour polymorphism make *C. carnea* an excellent model with which to investigate floral exploitation of the visual system and behaviour of bee pollinators. Due to the lack of any obvious resemblance to nectar-producing models, it has been suggested that most food-deceptive *Caladenia* species, including *C. carnea*, are likely to be using non-model mimicry to attract generalist food-seeking insects (Phillips et al. 2009; but see Dixon and Christenhusz 2018).

In the current study, we first used laboratory-based testing of sugarbag bees to investigate if previously observed innate preferences for UV-absorbing white artificial stimuli might apply when considering colour morphs of cultivated *C. carnea* orchid flowers sourced from the Australasian Native Orchid Society. In our lab-based study, we additionally tested if the dorsal sepal that reflected UV radiation influenced bee choices if the UV signal was blocked. Our analyses of spectra also considered modulation of green receptor contrast, since that factor has previously been shown to be important for colour choices by sugarbag bees (Dyer et al. 2016a). Second, we waited for the *C. carnea* orchid flowers to bloom at a native field site to collect additional spectral data in situ, to enable an understanding of how the lab-based study might apply in a more natural context.

Materials and methods

Laboratory conditions for testing innate preferences

The innate colour preferences of the sugarbag bee were tested in a controlled temperature (CT) room (3 m × 5 m) at Monash University during 2009. The room was illuminated by four Philips Master TLS HE slimline 28 W/865 UV + daylight fluorescent tubes (Philips Holland) with specially fitted high-frequency (1200 Hz) ATEC Jupiter EGF PMD2614-35 electronic dimmable ballasts. The illumination was diffused by Rosco 216 (Germany) UV-transmitting screen and approximately matched daylight illumination conditions for foraging bees (Dyer and Chittka 2004).

The CT room allowed temperature to be adjusted, which enabled control of the bees to return to their nesting box (28 × 20 × 31 cm (LWH)) when the ambient temperature was lowered to 19 °C (Norgate et al. 2010; Spaethe et al. 2014). Actual experiments were conducted at 27 °C and 30% relative humidity (SPER-Scientific Hygrometer, Arizona, USA) to allow effective foraging activity of the bees (Norgate et al. 2010).

The bee colony used in the study was propagated by Dr Tim Heard (Sugarbag bee Australia) following established protocols (Heard 1988) so that a split hive contained new bees that were naïve with respect to flower stimuli. Pollen grains were provided directly to the nest box containing the bees. The nest box was connected by a Plexiglas tube with vertically lifting gates to one of two identical foraging arenas of dimensions 1.2 × 0.6 × 0.5 m (LWH). The arena sides contained lifting flaps to allow the easy exchange of stimuli. The arena lid was constructed of UV-transparent Plexiglas and conditions were identical to the previously reported experiments on innate preferences of sugarbag bees for artificial colour stimuli (Dyer et al. 2016a). Two arenas were used to allow for bee maintenance and arena cleaning during different phases of the experiment. Bees were allowed a minimum of 7 days to habituate to the laboratory conditions before any testing. During this time, three Plexiglas gravity feeders (Whitney et al. 2008) were placed at random coordinates within the arena providing 5% (vol.) sucrose solution ad libitum. Depleted feeders were removed and replaced with fresh feeders introduced at different locations every 2 h between the hours of 0900 and 1700, which corresponds with the peak foraging time of the bees (Heard and Hendrikz 1993). Previous experiments confirmed that after 1 week of habituation in the CT room, bee flight activity closely matched that of hives maintained outdoors (Heard and Hendrikz 1993; Norgate et al. 2010).

Flower stimuli

Caladenia carnea flowers were cultivated plants supplied by Richard Austin and Russell Mawson from the Australasian Native Orchid Society (ANOS Victorian Group) in 2009. The reflections of the flowers were measured from 300 to 700 nm using a spectrophotometer (S2000) with a PX-2 pulsed xenon light source attached to a PC running SPECTRA SUITE software (Ocean Optics Inc., Dunedin, FL, USA) and calibrated against a UV-reflecting PTFE white standard (Ocean Optics). Since this procedure indicated that the dorsal sepal of both the white and pink morphs of the flowers reflected UV radiation, a behavioural test of potential preferences for UV reflectance was also conducted using the white morph flower.

A goal of the study was to build a bridge between our current understanding of how colour choices are made by bees in a laboratory conditions for artificial stimuli (Dyer et al. 2016a), and how this might inform how flowers evolve in more natural contexts (Shrestha et al. 2019a, b). In 2018, additional spectral measurements of *C. carnea* flowers were taken at Baluk William Flora Reserve (37°5532S, 145°2045E), 40 km southeast of Melbourne, Victoria, Australia.

The spectral reflectance functions for both the cultivated and field collected *C. carnea* flowers were separately analysed and plotted in a hexagon colour space (Chittka 1992) using the methodology previously employed to model sugarbag bee colour perception in identical conditions (Spaethe et al. 2014; Dyer et al. 2016a, b), and data were interpreted in relation to recently determined colour discrimination functions for sugarbag bees (Spaethe et al. 2014; Garcia et al. 2017).

To enable behavioural testing, stimuli were created by encapsulating a cultivated flower within a 5 cm high and 5 cm diameter flower container covered by UV-transparent Glad WrapTM (The Clorox Company, Oaklands, CA, USA) to exclude olfactory cues that are known to influence the choice behaviour of bees (Giurfa et al. 1994; Kunze and Gumbert 2001; Kantsa et al. 2017). The base of the inflorescence was wrapped in moist tissue to preserve the flower. This allowed for systematic variation of visual factors mediating bee choices, whilst excluding olfactory confounds, and also minimised the impact on the number of flowers required for testing. This was important as the flowers are difficult to source, but it is of high value to understand the system to help manage wild-type populations when they are in flower. Following each test, the UV-transparent Glad Wrap was replaced, and the flowers were also replaced if there was any sign of wilting or damage from the manipulations.

Innate preferences of sugarbag bees for *C. carnea* flowers

As bees do not land on colour stimuli excluding scent in a laboratory setting unless pre-training is provided (Giurfa et al. 1995; Raine and Chittka 2007; Dyer et al. 2016a), the sugarbag bees were initially pre-trained to collect 10 µl droplets of 15% vol. sucrose solution placed in a small recessed well in the centre of three sandblasted aluminium disks (25 mm diameter) that reflected 300–700 nm radiation equally at about 25%, which has previously been successfully used to measure innate colour responses in sugarbag bees (Dyer et al. 2016a). The aluminium disks were mounted on clear plastic cylinders of 100 mm height and bees were allowed a minimum of 2 h to forage on the pre-training disks prior to an experiment. The disks were regularly cleaned and replaced when sucrose was depleted to minimise scent-based recruitment that is well known in stingless bees (Roselino et al. 2016). After pre-training, the temperature of the CT lab was lowered so that bees returned to the colony, the arena could be cleaned, and flower stimuli prepared in the flight arena.

To conduct a test, approximately 40 bees were first isolated in the plexiglass tube, and then the gate to the arena was opened to allow the bees to start foraging. The dependent variable was the number of touches to the flower container in the respective experiments detailed below. A maximum of one landing (clear contact with a stimulus) was scored per approach to a stimulus by a bee. No bee ever immediately (within 10 s) returned to a stimulus after having flown away. There was no observation of multiple visits where follower bees landed in quick succession on the same stimulus, suggesting data were independent and driven by individual colour visual choices rather than social cues. At the completion of each replicate, all the bees in a particular trial were sacrificed to avoid pseudo-replication. As so little is known about how stingless bee innate preferences may operate with respect to real flowers, the following experiments proceeded in a sequential fashion to map how bees may use their vision when foraging amongst flowers of different appearances.

The first behavioural experiment tested if bees had a significant preference for either the white or pink morphs of the *C. carnea* flowers. The two respective flowers were placed in the arena separated by 10 cm, thus presenting a dual choice scenario with respect to the acuity of sugarbag bees (Dyer et al. 2016b). The flower morphs were matched in size, and the location within the arena was randomised with respect to overall position, as well as the relative location of the white or pink morphs. When the bees were allowed to enter the arena by opening a vertical gate, the number of landings on the respective flower stimuli was scored for 5 min as a measure of preference for each of the two flower colours. We carried out 16 replicates, with a different set of bees per replicate.

The second behavioural experiment investigated the response of sugarbag bees towards a novel flower after having habituated to a previous flower. In this experiment, bees were presented with two flowers, one at a time. During the first presentation, a single flower was positioned at a random position in the arena for a period of 25 min, after which the number of landings was quantified for a 5 min period. Following this, the original flower stimulus was removed, and replaced with a second flower stimulus. We then immediately quantified, over a second 5 min observation period, the number of landings on the new flower stimulus. This protocol allowed us to compare the number of landings on the first and second flower. Bees were subjected to one of the four experimental treatments, involving manipulation of the colour of the flowers that were provided to bees during the first and second flower presentation: white–white, pink–pink, white–pink, and pink–white. The experiment involved eight replicates per treatment, with a different set of bees used per replicate.

The third experiment used a manipulation of the UV reflection on the dorsal sepal of the white morph of the flowers to test if this spectral information may be part of a visual signal used to attract or deter bees. Bees were presented with two flowers in a setup identical to the one described for the first experiment, with the exception that bees were presented with two flowers of the same colour (white), one with the UV signal present and one with the UV signal removed. The latter was achieved by applying a thin layer of sunscreen (Hamilton SPF 30 +, Adelaide, SA, Australia) over the dorsal sepal, as previously employed to modulate UV signals in flowers by Peter and Johnson (2008). The sunscreen had a cutoff wavelength of 399 nm and was thus well suited for modulating the UV signal of the flower petals (see electronic supplementary data at FigShare https://figshare.com/s/7f0bc21c9b3f8a592157). In this experiment, 16 replicates were performed, with a different set of bees per replicate.

To enable a quantitative statistical analysis for the effect of the different experimental manipulations on the number of observed landings of sugarbag bees, we formulated a separate generalised linear model (GLM) for each behavioural experiment using the number of landings as the response variable. For most of the analyses, the number of landings was analysed assuming a Poisson distribution to account for the discrete nature of the response variable (Zuur et al. 2009). However, for analysing the number of landings corresponding to the preference experiment (experiment one), a negative binomial distribution was assumed, as the preliminary models assuming a Poisson distribution were found to be overdispersed (overdispersion = 1.95). For the analysis of the second experiment, given that bees within each replicate were subjected sequentially to two sets of observations, a generalised linear mixed model (GLMM) was fitted to the data including a random term to account for this repeated measurement (Zuur et al. 2009). Analyses were done using the routines glm and glmer available as part of the base and lme4 (Bates et al. 2015) packages for the *R* statistical language for statistical computing release 3.5.1 (*R* Team, 2018).

Scanning electron microscopy (SEM) imaging

As the flowers showed evidence of different reflectance properties, epidermal surfaces were investigated following the technique described by van der Kooi et al. (2014). In brief, sepals and petal were pressed in dental impression material that solidifies within minutes. Positive surface replicates were subsequently generated by filling the mould with transparent nail polish, creating a cast. The casts were sputtered gold coated and images were acquired using a scanning electron microscope (Philips XL30) at the RMIT Microscopy and Microanalysis Facility (RMMF), RMIT University, Melbourne, Australia. We used 30 kV current with spot size five and magnification 1500X–1800X in 10 mm working distance from sample to current beam.

Results

Spectrophotometry of both cultivated (Fig. 1) and field (Fig. 2) *C. carnea* flowers revealed that the lateral sepals and petals of both white and pink morphs were UV absorbing (non-UV reflective), whilst the dorsal sepal of both flower morphs reflected UV radiation. Flower spectra for the cultivated plant flowers (Fig. 1a, b) and the field flowers (Fig. 2a, b) were plotted in a hexagon colour space (Fig. 1c, 2c) and colorimetry was informed by modelling of psychophysics data for sugarbag bees (Spaethe et al. 2014; Garcia et al. 2017) to provide probabilities of discrimination (Tables 1, 3). For the white-cultivated versus pink-cultivated flower lateral sepals (Table 1), the mean colour distance was 0.062 hexagon units, which is predicted to be poorly discriminated by sugarbag bees (56.8% accuracy). For the cultivated flowers, the UV-reflecting dorsal sepal was predicted to be discriminable from the rest of the white flower with and accuracy of 85.6% (colour distance 0.214 hexagon units), as was the UV-reflecting dorsal sepal for the pink flower morph (colour distance 0.359 hexagon units, predicted discrimination accuracy of 88.2%). Thus, modelling suggested the white and pink flower morphs could be discriminated from

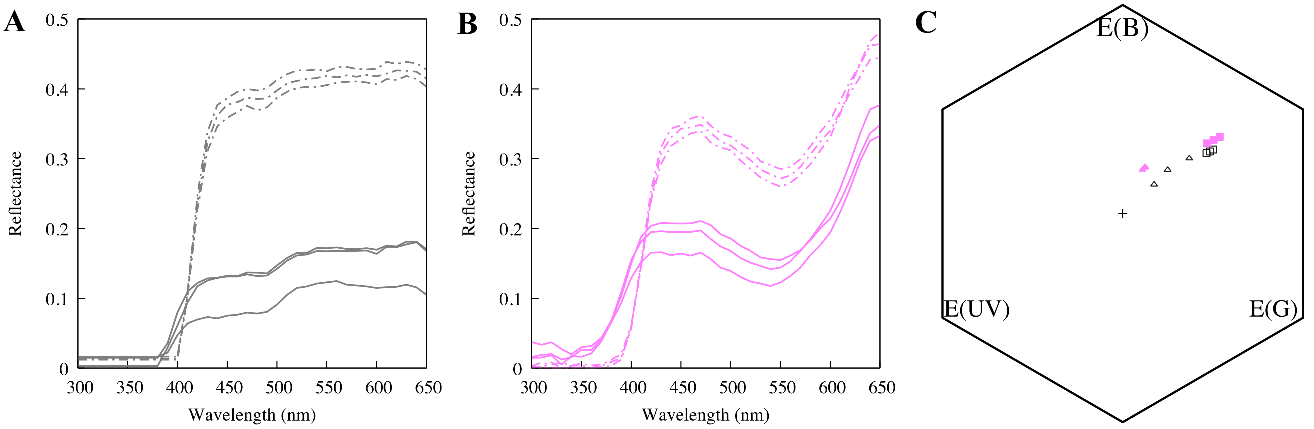

Fig. 1 Spectral reflectance of the dorsal sepal (solid lines) and lateral sepal (dash dotted line) of $n=3$ white (panel **a**.) and $n=3$ pink (panel **b**.) morphs of *Caladenia carnea* obtained from the Australasian Native Orchid (ANO) society. Panel **c** represents the spectra in panels **a** and **b** in the hexagon colour model of Chittka (1992). In the hexagon, the dorsal sepals are represented by triangle markers, whilst the lateral sepals are indicated by square markers. Empty markers correspond to the white morph and solid markers to the pink morph

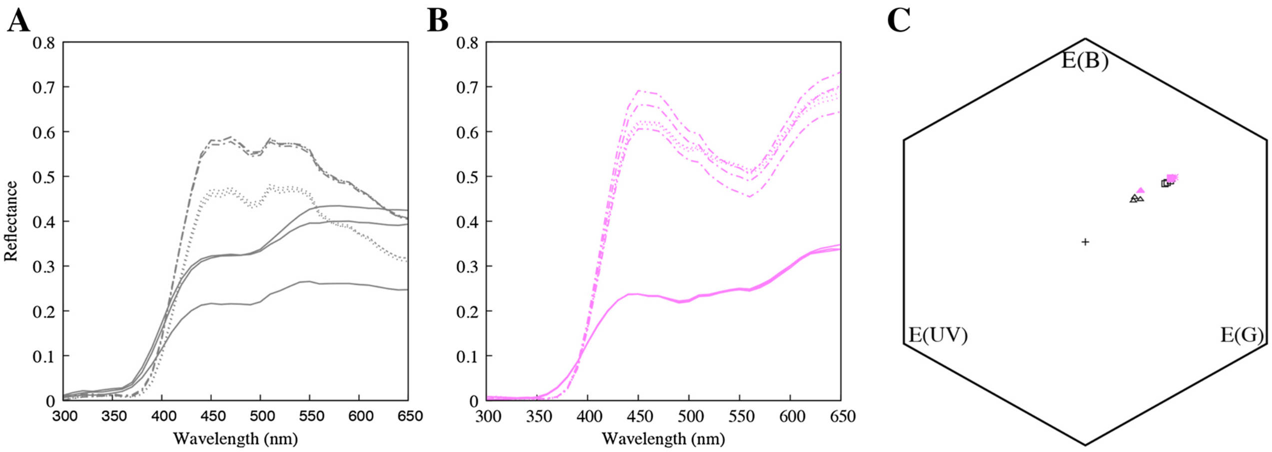

Fig. 2 Spectral properties of **a**. white and **b**. pink morphs of *Caladenia carnea* orchids measured in the field. Panels **a** and **b** show the reflectance spectral profiles for the dorsal (solid line) and lateral (dashed-dotted line) sepals, and petals (dotted line) of $n=3$ white and $n=3$ pink *C. carnea* orchids, respectively. Panel **c**. shows the spectra in panels **a** and **b** in the hexagon colour model of Chittka (1992). In the hexagon, the dorsal sepals are represented by triangle markers, the lateral sepals by square markers and the petals by asterisk. Empty, black markers correspond to the white morph and solid, pink markers to the second morph

Table 1 Euclidean distances calculated using the hexagon colour space (Chittka 1992) representing colour difference between samples obtained from white (W) and pink (P) morphs of $n = 3$ *Caladenia carnea* flowers obtained from the Australasian Native Orchid Society

	AGL	WDS	WLS	PDS	PLS
AGL	0.000				
	0.500				
WDS	0.305 ± 0.105	0.000			
	0.882 ± 0.000	*0.500*			
WLS	0.513 ± 0.018	0.214 ± 0.092	0.000		
	0.882 ± 0.000	*0.856 ± 0.050*	*0.500*		
PDS	0.240 ± 0.009	0.142 ± 0.060	0.326 ± 0.017	0.000	
	0.882 ± 0.000	*0.829 0.050*	*0.882 ± 0.000*	*0.500*	
PLS	0.560 ± 0.034	0.256 ± 0.096	0.062 ± 0.021	0.359 ± 0.032	0.000
	0.882 ± 0.000	*0.876 ± 0.015*	*0.568 ± 0.115*	*0.882 ± 0.000*	*0.500*

Measurements correspond to two different flower parts: dorsal sepal (DS) and lateral sepal (LS). All distances were calculated relative to a typical, foliage adaptation background (AGL) consisting of an average of Australian native green leaves (Bukovac et al. 2017). Values in italics represent the probability of discriminating the colour difference between two stimuli as predicted by the colour discrimination function for the appropriate region of colour space proposed by (Garcia et al. 2017) for *Tetragonula carbonaria*. All reported values are the mean of three measurements ± one standard deviation. Colour differences and associated discrimination probabilities for each individual measurement are provided in the data repository FigShare: https://figshare.com/s/7f0bc21c9b3f8a592157. Note that the highest probability shown (0.882) is based on empirical data for the highest frequency of correct choices by free flying *T. carbonaria* bees (Garcia et al. 2017). A probability of 0.500 indicates random choices for identical stimuli in a dual choice discrimination task

each other, and the dorsal sepal was also a distinctly different signal for each flower morph (Table 1). To further test if the above predictions might apply to other hymenopteran species considering an alternative colour model, we additionally consider an implementation of the receptor noise limited model for the honeybee *Apis mellifera* as high-quality photoreceptor data and receptor noise measurements exist for this species (Vorobyev and Osorio 1998; Hempel de Ibarra et al. 2014, Vorobyev et al. 2001). Although the honeybee was originally introduced to Australia in the 19th century, it is now considered an important pollinator, and has been observed at our field site, including a sighting (MS) of a honeybee visiting *C. carnea* flowers. For the cultivated flowers, the receptor noise modelling predicts that all flower spectra are discriminated above the threshold considering the visual capabilities of honeybees (Table 2). As the cultivated flowers were used in the behavioural experiments, those data are presented next in results. When the sugar bag bees were given the choice between a white- and pink-cultivated orchid flower (Experiment 1), we found that they significantly preferred the white flowers (Deviance $(G) = 8.68$, $P = 0.003$; Fig. 3). When bees were presented with flowers sequentially (Experiment 2), we found no significant difference in the number of landings for changes between flowers for the cases of white–white ($G = 1.26$, $P = 0.262$), pink–pink ($G = 1.40$, $P = 0.237$) (Fig. 4a, b), nor was there a significant change between preferred (white) to non-preferred (pink) flowers (Fig. 4c) ($G = 3.05$, $P = 0.081$). Intriguingly however, we found a significant preference in the number of landings when bees sequentially switched from non-preferred (pink) to preferred (white) colours ($G = 19.0$, $P < 0.001$, Fig. 4d). Finally, in the experiment that tested for a possible effect of a preference for white flowers containing a UV-reflecting dorsal sepal compared to a dorsal sepal with ablated UV reflectance (Experiment 3), we found a significant preference for the flowers with a UV-reflective dorsal sepal ($G = 33.9$, $P < 0.001$, Fig. 5).

Comparing the white and pink morphs of naturally growing *C. carnea* flowers sampled in 2018, the mean colour distance of white versus pink lateral sepals (Table 3) was 0.025 hexagon units, which is predicted to not be discriminated by sugarbag bees (50% accuracy). By contrast, the UV-reflecting dorsal sepal was predicted to be discriminable from the rest of the white flower with greater than 80% discrimination accuracy, as was the UV-reflecting dorsal sepal for the pink flower morph. To further test if the above predictions might apply to other hymenopteran species considering an alternative colour model, we found that the receptor noise limited model for the honeybee predicts that all respective flower spectra are also discriminated above threshold considering the visual capabilities of honeybees (Table 4).

As green receptor contrast is important for flower preferences in sugarbag bees (Dyer et al. 2016a), we additionally calculated excitation values for all respective flower regions, the data for which are presented in Table 5.

Scanning electron microscopy (SEM) imaging

Initial observations of the surfaces of the different floral parts for the two colour morphs showed that there are

Table 2 Theoretical receptor noise (RN) colour model values (Vorobyev and Osorio 1998) representing colour difference between samples obtained from white (W) and pink (P) morphs of $n=3$ *Caladenia carnea* flowers obtained from the Australasian Native Orchid Society

	AGL	WDS	WLS	PDS	PLS
AGL	0.000				
	0.500				
WDS	7.13 ± 2.03	0.000			
	1.00 ± 0.000	*0.500*			
WLS	10.7 ± 0.232	3.69 ± 1.76	0.000		
	1.00 ± 0.000	*0.995 ± 0.009*	*0.500*		
PDS	7.03 ± 0.056	3.93 ± 0.859	6.45 ± 0.218	0.000	
	1.00 ± 0.000	*1.00 ± 0.000*	*1.00 ± 0.000*	*0.500*	
PLS	11.3 ± 0.391	4.21 ± 1.72	1.67 ± 0.068	6.05 ± 0.388	0.000
	1.00 ± 0.000	*1.00 ± 0.000*	*0.991* ± 0.002	*1.00* ± 0.000	*0.500*

Receptor noise values were calculated using peak photoreceptor values from (Peitsch et al. 1992) and noise parameters for *Apis mellifera* reported by Vorobyev and Osorio (1998). Measurements correspond to two different flower parts: dorsal sepal (DS) and lateral sepal (LS). All distances were calculated relative to a typical, foliage adaptation background (AGL) consisting of an average of Australian native green leaves (Bukovac et al. 2017). Values in italics represent the probability of discriminating the colour difference between two stimuli as predicted by the 'blue' colour discrimination function proposed by (Garcia et al. 2017) for *Apis mellifera*. All reported values are the mean of three measurements ± one standard deviation. Colour differences and associated discrimination probabilities for each individual measurement are provided in the data repository FigShare: https://figshare.com/s/7f0bc21c9b3f8a592157. Note that the highest probability shown (1.000) is based on empirical data for the highest frequency of correct choices by free flying *A. mellifera* bees (Garcia et al. 2017). A probability of 0.500 indicates random choices for identical stimuli in a dual choice discrimination task

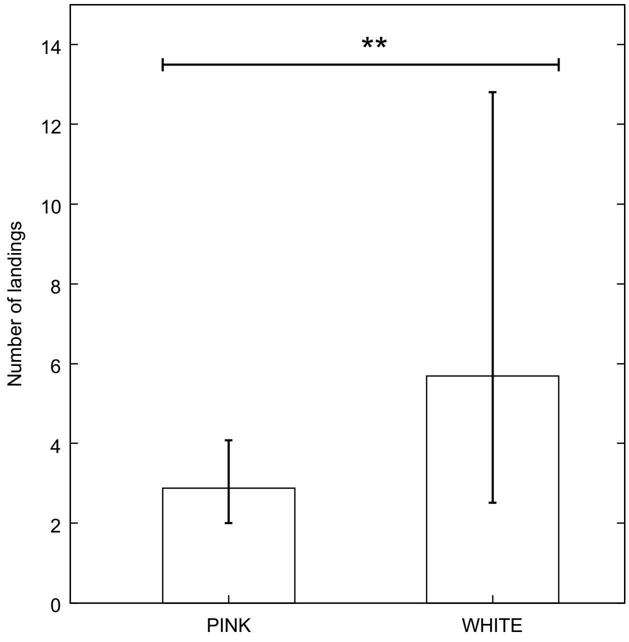

Fig. 3 Mean number of landings observed for the pink ($n=16$ bees) and white morphs ($n=16$ bees) of C. carnea. **$P<0.01$. Error bars represent 95% confidence interval (CI) for the respective mean number of landings

morphological differences between the dorsal sepal, lateral sepal and petal. There are, however, no marked differences in surface shape between the two colour morphs (Fig. 6), and the petal cell shape would produce a matt-type reflection (van der Kooi et al. 2019).

Discussion

The colour preferences of important pollinators like bees have been thought to be an important factor in how flowering plants may initially receive visits in a way that promotes efficient pollination (Darwin 1877; Giurfa et al. 1995; Raine and Chittka 2005; Raine et al. 2006; Raine and Chittka 2007; Ings et al. 2009; van der Kooi et al. 2019). However, clear evidence of how this may apply in natural environments for real flowers is a difficult problem to approach (Dyer et al. 2007). Here, we show that sugarbag bees demonstrated a significant preference for white *C. carnea* flower morphs compared to pink flower morphs (Fig. 3), which is consistent with our colorimetric modelling data that the cultivated flowers can be reliably discriminated (Tables 1, 2), and also that the white morph lateral sepals modulated the green receptor channel to a greater extent (Table 5). From the standpoint of the respective orchid flower type, such colour preferences by sugarbag bees could potentially result in fitness differences for a particular orchid depending on the colour of its flower. It is also possible that negative frequency-dependent selection may be important, with pollinator behaviour towards rewardless orchid flowers favouring the rarer colour morph and, in so doing, helping to maintain floral colour variation (Smithson and Macnair 1997). Such

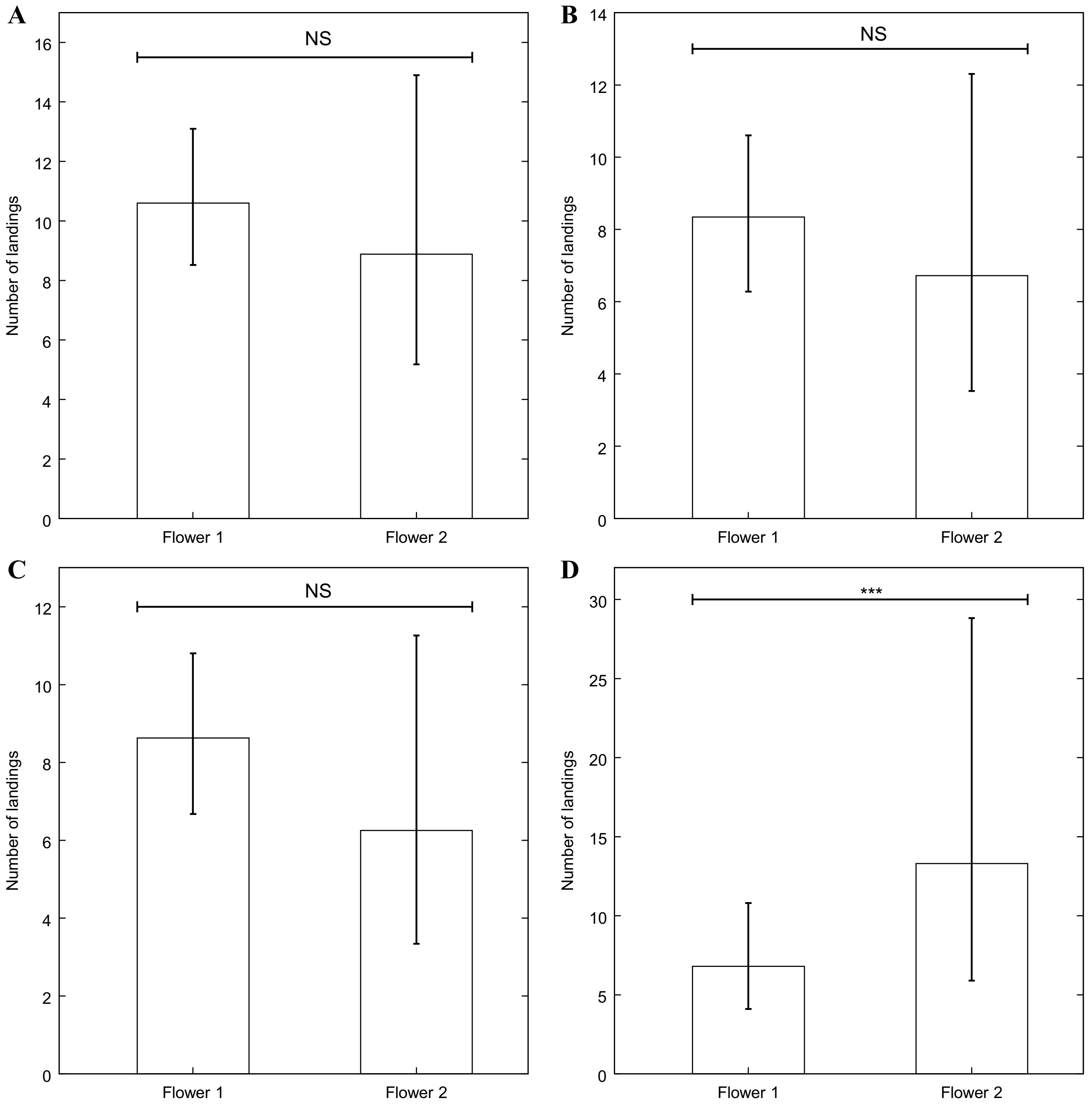

Fig. 4 Results of the habituation experiments. Number of landings observed for the first and second flower presentation for pink (pink columns) and white (white columns) flowers ($n = 8$ bees on each experiment). ***$P < 0.001$. Error bars represent 95% confidence interval (CI) for the respective mean number of landings

negative frequency-dependent variation was demonstrated in the food-deceptive European orchid *Dactylorhiza sambucina* (Gigord et al. 2001).

Previous work on both sexually (Wong and Schiestl 2002; Wong et al. 2004; Gaskett 2011; Stejskal et al. 2015) and food-deceptive (Dafni 1984; Smithson and Gigord 2003) orchids suggests that Hymenopterans habituate to a particular flower type that provides no reward, which is also consistent with well-known associative learning mechanisms in bees (Gumbert 2000; Simonds and Plowright 2004). For example, in sexually deceptive orchids, male Hymenopterans quickly learn the presence of unrewarding flowers, and subsequently avoid flowers along with locations where they had previously been deceived (Wong and Schiestl 2002; Wong et al. 2004). In theory, such habituation could act as a selective force against non-rewarding flowers. Despite this,

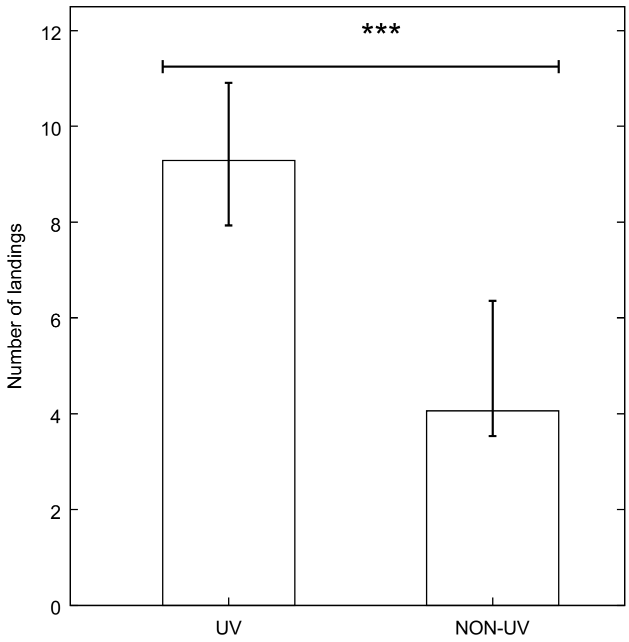

Fig. 5 Mean number of landings observed on flowers with unaltered UV-reflective sepals (violet column, $n=16$ bees) and UV ablated sepals (grey column, $n=16$ bees). ***$P<0.001$. Error bars represent 95% confidence interval (CI) for the respective mean number of landings

orchids are a highly evolved and very successful plant group within the angiosperms (Nilsson 1992; Dressler 1993), and one possibility for the success of rewardless orchids could be the ability to alter floral signals to avoid habituation effects (Juillet and Scopece 2010). Sexually deceptive Ophrys heldreichii orchid flowers avoid this habituation effect, for example, by displaying novel patterns that bees perceive as different, which can promote transfer of pollinia between conspecific flowers (Stejskal et al. 2015). Currently, little is known about how novel flower colours of orchid morphs may benefit from cross-pollination; psychophysics and field experiments suggest bees have a preference for visiting discriminable but similar colours rather than distinctly different colours (Peter and Johnson 2008; Dyer and Murphy 2009). In our habituation experiments, we were thus interested to know if innate preferences might play a role in how bees choose between the different flower morphs of *C. carnea* orchids. Intriguingly, we found a significant increase in the number of landings to a newly introduced white flower that sugarbag bees innately preferred (Figs. 3, 4), thus countering any habituation effect towards unrewarding orchids. It seems reasonable, therefore, that the existence of multiple flower colours in morphs of *C. carnea* could have fitness consequences for the orchid by making it more difficult for their pollinators to associate a particular colour with non-rewarding flowers, which provides a plausible explanation

Table 3 Euclidean distances in the hexagon colour space (Chittka 1992) representing colour difference between samples obtained from white (W) and pink (P) morphs of $n=3$ *Caladenia carnea* flowers collected in the field

	AGL	WDS	WLS	WP	PDS	PLS	PP
AGL	0						
	0.500						
WDS	0.320 ± 0.015	0					
	0.882 ± 0.000	*0.500*					
WLS	0.490 ± 0.015	0.172 ± 0.019	0				
	0.882 ± 0.001	*0.879 ± 0.003*	*0.500*				
WP	0.515 ± 0.001	0.197 ± 0.014	0.026 ± 0.012	0			
	0.882 ± 0.000	*0.881 ± 0.001*	*0.500 ± 0.000*	*0.500*			
PDS	0.363 ± 0.003	0.047 ± 0.009	0.136 ± 0.013	0.160 ± 0.003	0		
	0.882 ± 0.000	*0.500 ± 0.000*	*0.866 ± 0.009*	*0.878 ± 0.001*	*0.500*		
PLS	0.513 ± 0.001	0.194 ± 0.013	0.025 ± 0.011	0.006 ± 0.001	0.156 ± 0.003	0	
	0.882 ± 0.000	*0.881 ± 0.001*	*0.500 ± 0.000*	*0.500 ± 0.000*	*0.877 ± 0.001*	*0.500*	
PP	0.524 ± 0.010	0.206 ± 0.016	0.034 ± 0.015	0.009 ± 0.008	0.169 ± 0.009	0.014 ± 0.007	0
	0.882 ± 0.000	*0.881 ± 0.000*	*0.500 ± 0.000*	*0.500 ± 0.000*	*0.879 ± 0.000*	*0.500 ± 0.000*	*0.500*

Measurements correspond to three different flower parts: dorsal sepal (DS), lateral sepal (LS) and petals (P). All distances were calculated relative to a typical, foliage adaptation background (AGL) consisting of an average of Australian native green leaves (Bukovac et al. 2017). Values in italics represent the probability of discriminating the colour difference between two stimuli as predicted by the colour discrimination function for the appropriate region of colour space proposed by (Garcia et al., 2017) for *Tetragonula carbonaria*. All reported values are the mean of three measurements ± one standard deviation. Colour differences and associated discrimination probabilities for each individual measurement are provided in the data repository FigShare: https://figshare.com/s/7f0bc21c9b3f8a592157. Note that the highest probability shown (0.882) is based on empirical data for the highest frequency of correct choices by free flying *T. carbonaria* bees (Garcia et al. 2017). A probability of 0.500 indicates random choices for identical stimuli in a dual choice discrimination task

Table 4 Theoretical receptor noise (RN) colour model values (Vorobyev and Osorio 1998) representing colour difference between samples obtained from white (W) and pink (P) morphs of $n = 3$ *Caladenia carnea* flowers collected in the field

	AGL	WDS	WLS	WP	PDS	PLS	PP
AGL	0.000						
	0.500						
WDS	7.32 ± 0.158	0.000					
	1.00 ± 0.000	*0.500*					
WLS	10.4 ± 0.151	3.17 ± 0.214	0.000				
	1.00 ± 0.000	*1.00 ± 0.000*	*0.500*				
WP	10. ± 0.014	3.36 ± 0.189	0.289 ± 0.116	0.000			
	1.00 ± 0.000	*1.00 ± 0.000*	*0.666 ± 0.075*	*0.500*			
PDS	8.02 ± 0.050	0.961 ± 0.167	2.65 ± 0.148	2.88 ± 0.046	0.000		
	1.00 ± 0.000	*0.929 ± 0.027*	*0.999 ± 0.000*	*1.00 ± 0.000*	*0.500*		
PLS	10.9 ± 0.047	3.57 ± 0.105	0.807 ± 0.049	0.940 ± 0.049	2.88 ± 0.058	0.000	
	1.00 ± 0.000	*1.00 ± 0.000*	*0.900 ± 0.013*	*0.930 ± 0.009*	*1.00 ± 0.000*	*0.500*	
PP	10.8 ± 0.083	3.51 ± 0.157	0.467 ± 0.084	0.528 ± 0.038	2.92 ± 0.087	0.435 ± 0.032	0.000
	1.00 ± 0.000	*1.00 ± 0.000*	*0.770 ± 0.041*	*0.801 ± 0.018*	*1.00 ± 0.000*	*0.755 ± 0.018*	*0.500*

Receptor noise values were calculated using peak photoreceptor values from (Peitsch et al. 1992) and noise parameters for *Apis mellifera* reported by Vorobyev and Osorio (1998). Measurements correspond to three different flower parts: dorsal sepal (DS), lateral sepal (LS) and petals (P). All distances were calculated relative to a typical, foliage adaptation background (AGL) consisting of an average of Australian native green leaves (Bukovac et al. 2017). Values in italics represent the probability of discriminating the colour difference between two 'blue' stimuli as predicted by the colour discrimination function proposed by (Garcia et al. 2017) for *Apis mellifera*. All reported values are the mean of three measurements ± one standard deviation. Colour differences and associated discrimination probabilities for each individual measurement are provided in the data repository FigShare: https://figshare.com/s/7f0bc21c9b3f8a592157. Note that the highest probability shown (1.000) is based on empirical data for the highest frequency of correct choices by free flying *A. mellifera* bees (Garcia et al. 2017). A probability of 0.500 indicates random choices for identical stimuli in a dual choice discrimination task

for how these food-deceptive flowers achieve pollination, and why distinct morphs of the same species exist in the same habitat.

Another important question about the visual signalling of *C. carnea* orchids came from the spectrophotometer evidence that the dorsal sepal of the flower reflected a UV signal. A colorimetric analysis (Table 1) of the data in a hexagon colour space suggested that the dorsal sepal signal was distinct both from the background and also the rest of the flower (Fig. 1). The UV reflectance is interesting in that it only partially extends into the UV (Fig. 1), thus potentially providing an attractive (Heiling et al. 2003) and novel signal, whilst avoiding making the sepal achromatic to bee vision (Dyer et al. 2007; Lunau et al. 2011). When the preferences of sugarbag bees were tested with flowers that either included the natural UV signal versus those in which the UV signal had been experimentally removed, bees showed a significant preference for choosing the wild-type flowers that contained the UV signal (Fig. 5). Intriguingly, predatory Australian crab spiders, *Thomisus spectabilis*, also appear to use UV reflection to attract ultraviolet-sensitive bees to flowers (Heiling et al. 2003). Whilst the reflection of a UV signal from the dorsal sepal of *C. carnea* orchids is unlikely to be as dire for unsuspecting flower visitors as crab spiders, this type of visual signal appears to be a useful way to drive innate preferences in bees, including the sugarbag bees.

Table 5 Mean and ± standard deviation of green receptor excitation values [E(G)] calculated from reflectance values of dorsal (DS) and lateral (LS) sepals of the white (W) and pink (P) morphs of three *Caladenia carnea* orchids provided by the Australian Native Orchid Society (ANO), and three samples collected in the field

Petal region	ANO E(G)	FIELD E(G)
WDS	0.484 ± 0.045	0.686 ± 0.045
WLS	0.731 ± 0.005	0.785 ± 0.001
WP	NR	0.747 ± 0.002
PDS	0.529 ± 0.024	0.624 ± 0.001
PLS	0.673 ± 0.007	0.787 ± 0.008
PP	NR	0.789 ± 0.001

Petal (P) reflectance was not recorded (NR) for orchids provided by ANO. Contrast is measured as the value difference to background excitation of 0.5 (Spaethe et al. 2001)

Together the behavioural findings for how sugarbag bees make choices towards *C. carnea* orchid flowers show that innate preferences—as are often observed in laboratory experiments to understand how bees perceive colour (Menzel 1967; Giurfa et al. 1995; Raine and Chittka 2005; Raine et al. 2006; Raine and Chittka 2007; Ings et al. 2009; Morawetz et al. 2013, Dyer et al. 2016a)—are

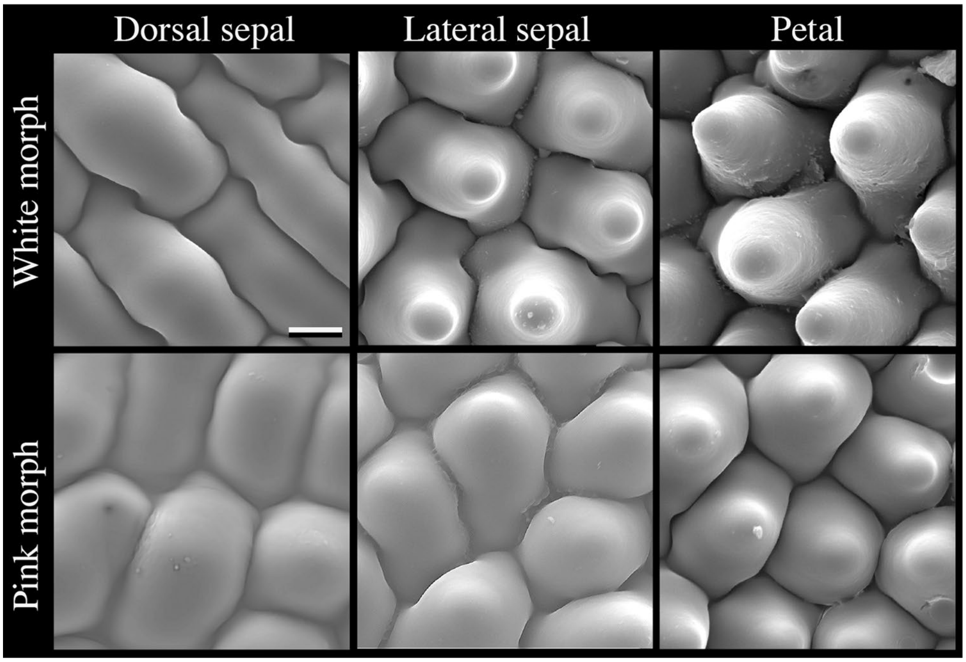

Fig. 6 Floral sepal and petal surface micro-structure of the white (upper row) and pink (lower row) colour morphs *C. carnea*. Scale bar for all figures = 20 µm

biologically relevant to how bees may choose real flowers in a more natural, biological setting. This is important as there are increasing attempts in trying to understand how bee psychophysics may link to pollinator choices for real flowers, and how flowers evolve signals to enhance communication (van der Kooi et al. 2019). Our initial survey of the surface shape for both colour morphs revealed that the dorsal sepals are convex shaped (flat cell), whilst the lateral sepals and petals feature cone-shaped epidermal cells (Fig. 6). These two types of epidermal cell shapes are widespread throughout the plant kingdom (van der Kooi et al. 2014), and backscatter light in many directions thus producing a matte visual appearance (van der Kooi et al. 2014, 2017). Although surface shape is an important determinant for the directionality of the reflected light, the production of different UV signals is likely to be due to different floral pigments (van der Kooi et al 2019) and additional studies are needed to infer which pigments may be responsible for the observed differences in ultraviolet reflectance. It is nevertheless interesting that cell shape differences are observed within single *C. carnea* flowers. The cone-shaped surfaces are known to provide tactile cues or grip to pollinators that land and walk on the flower (Kevan and Lane 1985; Whitney et al. 2011), and such mechanical effects may explain the increase in epidermal cell shape observed for the lateral sepals and petals, which may be touched more by pollinators than the dorsal sepal (Fig. 6). Future work could try to understand whether different morphs of *C. carnea* have different types, amount and/or localisation of floral pigments (van der Kooi et al. 2016), and how these may interact with flower physiology to promote plant–pollinator interactions (van der Kooi et al. 2019).

Our field collected data on *C. carnea* white and pink morphs revealed both some similarities, and differences, in the cultivated flowers used for the laboratory-based behavioural experiments. For example, whilst the general flower spectra are of similar shape (Figs. 1, 2) and there was evidence in both groups of flowers that the dorsal sepal reflected UV and is always discriminable from the other flower parts, only the cultivated white versus pink morphs revealed a colorimetric difference between the dorsal sepals for the two morphs that was predicted to be perceivable considering hue and modelling in a colour hexagon for sugarbag bees (Table 1). In contrast, petals, dorsal and lateral sepals of the white and pink morphs of field-measured orchids had very similar hues which are unlikely to be discriminated by *T. carbonaria* (Table 3).

One of the difficulties in understanding how colour signals may evolve is that different descriptors of colour like hue, saturation, brightness or G-contrast may interact in complex ways (Giurfa et al. 1996; Koethe et al. 2016; Ng et al. 2018; van der Kooi et al. 2019), which was the case for our cultivated flowers where both variation in hue and/or modulation of green contrast may be consistent with observed preferential behaviour by sugarbag bees. However, given that for the available flowers collected from the field hue does not appear to be very important (Fig. 2), it might be reasonable to conclude that G-contrast may be a main factor mediating innate preferences in sugarbag bees. This would be consistent with the laboratory-based experiments on sugarbag bees with artificial flowers (Dyer et al.

2016a), and some experiments with male long-horned *Tetralonia berlandi* bees visiting manipulated *Ophrys heldreichii* orchid flowers (Streinzer et al. 2009).

Future work should consider the extent to which natural flower populations that are bee pollinated have signals that maximise green contrast, as we seek to understand the major factors potentially contributing to flower communities (Shrestha et al. 2019a, b). However, caution must always be emphasised in trying to understand colour vision, as several studies report that honeybees are poor at detecting stimuli even with high green contrast, unless there is also some modulation of chromatic contrast by stimuli (Giurfa et al. 1996; Ng et al. 2018). It is also important to consider that different bee species (or, indeed, other insect pollinators) may process visual information in different ways that can affect flower colour evolution (Garcia et al. 2017; Shrestha et al. 2019a, b), which may complicate analyses of specific plant–pollinator interactions. It is also possible that other processes like vegetative fitness (Jersáková et al. 2006), neutral evolution (Chittka et al. 2001) or thermal regulation (Shrestha et al. 2018) may influence spectral changes in ways that are not directly related to pollinator preferences, although in the current study the white *C. carnea* morph does have a spectrum that are consistent with signal evolution to promote bee pollination (Shrestha et al. 2013, 2019a). It is also possible that colour morphs could theoretically represent different pollination mechanisms that may have evolved independently, since within *Caladenia* there appears to be plants that employ either sexual deceit or rewarding mechanisms (Faast et al. 2009).

Our findings also suggest that it would be of value to build detailed surveys of the frequency of *C. carnea* white and pink morphs in different environments and potentially using multi-year ecological sampling techniques (Xu et al. 2011; Rakosy et al. 2012; Paudel et al. 2018, Tao et al 2018) to understand how observed pollinator preferences may influence flower communities. Building a stronger understanding of this system will provide important insights into the complexity of colour signal processing in plant–pollinator interactions. It is important to point out, however, that, in nature, the number of visits a non-rewarding orchid receives by naive pollinators is expected to depend on a range of ecological factors, such as flowering time and the frequency of rewarding plants that are flowering nearby. It is also important to point out that the current study intentionally only examined visual cues, but in natural settings, pollinators may obtain and assess information about their environment from a variety of visual and olfactory cues (Kantsa et al. 2017). The question of which cue has greater influence on pollinator decisions warrants further investigation (Kunze and Gumbert 2001; Leonard et al. 2011; Leonard and Masek 2014), and provides interesting avenues for future research.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval No ethical permission was required for bee experiments. All orchid samples were provided with appropriate authority.

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