

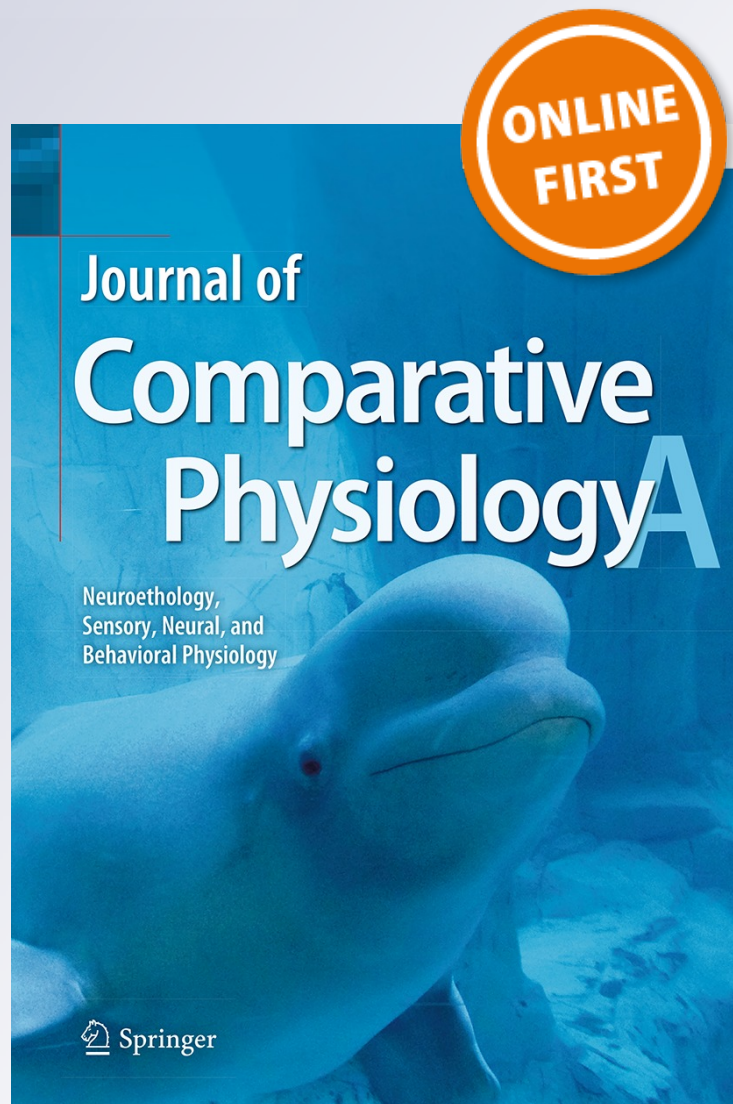
*Innate colour preferences of the Australian native stingless bee *Tetragonula carbonaria* Sm.*

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Innate colour preferences of the Australian native stingless bee *Tetragonula carbonaria* Sm.

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Abstract Innate preferences promote the capacity of pollinators to find flowers. Honeybees and bumblebees have strong preferences for ‘blue’ stimuli, and flowers of this colour typically present higher nectar rewards. Interestingly, flowers from multiple different locations around the world independently have the same distribution in bee colour space. Currently, however, there is a paucity of data on the innate colour preferences of stingless bees that are often implicated as being key pollinators in many parts of the world. In Australia, the endemic stingless bee *Tetragonula carbonaria* is widely distributed and known to be an efficient pollinator of both native plants and agricultural crops. In controlled laboratory conditions, we tested the innate colour responses of naïve bees using standard broadband reflectance stimuli representative of common flower colours. Colorimetric analyses considering hymenopteran

vision and a hexagon colour space revealed a difference between test colonies, and a significant effect of green contrast and an interaction effect of green contrast with spectral purity on bee choices. We also observed colour preferences for stimuli from the blue and blue–green categorical regions of colour space. Our results are discussed in relation to the similar distribution of flower colours observed from bee pollination around the world.

Keywords Vision · Flower · Insect · Pollinator · Southern hemisphere

Introduction

The biological partnership of bees and the flowers they visit to collect nutrition is a classic example of visual ecology that has been intensively investigated over the past century (von Frisch 1914; Lythgoe 1979; Barth 1985; Chittka et al. 2001; de Ibarra et al. 2014). Mainstream pollinator models including honeybees (Galizia et al. 2012; Avarguès-Weber and Giurfa 2014) and bumblebees (Goulson et al. 2007; Leonard et al. 2011) have provided significant insights into how sensory cues enable bees to reliably find flowers to collect nutrition (Leonard et al. 2011; Dyer et al. 2014), and incidentally transfer pollen to conspecific plant flowers (Proctor and Yeo 1973; Adler and Irwin 2006; Ballantyne et al. 2015). Chittka and Menzel (1992) established that the spectral profile of flowers in the Northern Hemisphere (Israel) frequently have signal characteristics that closely match the region of the spectrum where honeybees have colour discrimination maxima (von Helversen 1972). However, this close match of flower colour signals to bee vision is not an example of co-evolution since bee trichromatic vision is phylogenetically ancient and highly

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conserved (Peitsch et al. 1992; Chittka 1996; Briscoe and Chittka 2001), and thus, insect pollinated flowering plants often generate spectral signals that suit the colour capabilities of important bees, or other potential pollinators in an environment (Lunau et al. 2011; Shrestha et al. 2013).

For honeybees and bumblebees, there exist detailed data on how receptor signals facilitate colour processing at a neural level by opponent mechanisms in the brain (Kien and Menzel 1977; Yang et al. 2004; Paulk et al. 2009; Dyer et al. 2011) which facilitates trichromatic colour perception as demonstrated in behavioural experiments (von Frisch 1914; Daumer 1956; von Helversen 1972; Backhaus and Menzel 1987; Giurfa 2004; Dyer et al. 2008; Reser et al. 2012). This detailed knowledge has allowed for the development of colour space models (Backhaus and Menzel 1987; Chittka 1992; Vorobyev and Brandt 1997; Kemp et al. 2015) to facilitate analyses of how flower signals are distributed in different environments (Chittka et al. 1994; Chittka and Wells 2004). However, the distribution of flower colours in the Northern Hemisphere is not equally spread in bee colour space, showing certain peaks and troughs that likely represent the regions of the visual spectrum where important bee pollinators most efficiently process flower colour signals (Chittka et al. 1994; Chittka and Wells 2004; Lunau et al. 2011). In addition to spectral spacing of photoreceptors and subsequent neural processing (Chittka and Wells 2004; Dyer et al. 2011), another important factor that could influence flower spectral signal evolution (van der Kooi et al. 2016) is how certain pollinators may have innate colour preferences (Menzel 1967; Lunau 1990; Giurfa et al. 1995; Lunau et al. 1996; Kelber 1997; Gumbert 2000; Pohl et al. 2008; Morawetz et al. 2013; Lunau 2014; Telles et al. 2014), which was first proposed by Darwin (1877) as a potential mechanism to help inexperienced pollinators first find food.

Menzel (1967) showed in free flying honeybees that narrow band stimuli (413 nm) are learnt more rapidly than other longer wavelength spectral stimuli; and subsequent work reveals that under controlled experimental conditions naïve honeybees have innate preferences for 'blue' stimuli with a dominant wavelengths around 410–420 nm (Giurfa et al. 1995; Avarguès-Weber and Giurfa 2014). Such stimuli lie in a bee UV-blue categorical region of hexagon colour space (Chittka et al. 1994), and Giurfa et al. (1995) found that flowers that lie within this region of bee colour space had a tendency to contain higher nectar rewards.

Bumblebees also show innate colour preferences for certain stimuli based on a variety of potential factors like saturation (Lunau et al. 1996; Ings et al. 2009). In fact, in controlled lab experiments with real flowers like Snapdragons (*Antirrhinum majus*), naïve bumblebees preferred wild type flowers compared to mutants that lacked pigment colouration (Dyer et al. 2007). Chittka et al.

(2004) considered bumblebee perception using broadband colour stimuli and found a colour preference for bee 'UV-blue' stimuli and in the Wuerzburg region of Germany flowers with this characteristic having relatively higher levels of nectar reward. Indeed, bumblebees show a preference for blue stimuli across a wide geographic range (Chittka et al. 2004; Raine and Chittka 2005; Raine et al. 2006). However, in other important pollinating species like flower visiting flies, there is a preference for 'yellow' stimuli (Kugler 1950; Lunau and Wacht 1994, 1997), and some evidence suggest that these colour preferences in flies have influenced flower evolution (Kay 1976; Stanton et al. 1989; Dinkel and Lunau 2001).

In Australia, stingless bees are important pollinators (Armstrong 1979; White et al. 2001; Michener 2007) that have contributed to the evolution of flower signals in this large and geologically isolated island (McLoughlin 2001; Dyer et al. 2012; Shrestha et al. 2013). Surprisingly, when the spectra of Australian native flowering plants was evaluated using the methods employed for studying plant-pollinator interactions in the Northern Hemisphere (Chittka and Menzel 1992; Chittka et al. 1994), a very similar distribution of loci was observed in bee colour space (Dyer et al. 2012). Whilst there are many stingless bee species in Australia (Michener 2007), *Tetragonula carbonaria* (Smith 1854) (hereafter *T. carbonaria*) has been identified as an important model pollinator of flowering plants including agricultural crops (Heard 1994, 1999; Heard and Dollin 1998). *T. carbonaria*, previously known as *Trigona carbonaria*, is endemic to Australia (Green et al. 2001) and has the most widely known distribution of any native bee (Dollin et al. 1997; Dollin 2010; Halcroft et al. 2013). These bees usually choose large tree cavities to provide insulation for the colony, and have a reported range along the east coast as far north as the Atherton Tablelands in Queensland (17°15S) and as far south as Bega, in New South Wales (36°40S). *T. carbonaria* bees produce a type of 'Pot-honey' to store excess nectar collected from flowers (Halcroft et al. 2013) and forager bees thus act as central place foragers, allowing for experimental access to research questions to better understand plant-pollinator interactions (Dyer et al. 2012). These bees are active year round when temperature is greater than 18° (Heard and Hendrikz 1993), and typically forage as generalists that and can learn to collect nectar from a variety of flowers (Heard 1999). Recent work shows that colour perception of *T. carbonaria* can be tested in laboratory conditions (Spaethe et al. 2014), enabling a high level control over factors like light and temperature that easy influence stingless bee behaviour (Heard and Hendrikz 1993; Norgate et al. 2010).

Currently, we are unaware of any previous work testing the potential innate colour preferences of Australian native stingless bees. For innate colour preference testing,

it is necessary to ensure that test bees have had no prior experience with flowers, since learning may change colour perception (Giurfa et al. 1995; Raine and Chittka 2007; Avarguès-Weber and Giurfa 2014). We use broadband colour stimuli to test the innate colour preferences of specially reared *T. carbonaria* bees to obtain insights into whether these bees do show preferences for colour stimuli, and what potential factors of colour perception (Kemp et al. 2015) may influence bee decisions. We discuss our findings in relation to previous studies on the model bee species to try and build a bridge between classic bee studies, and more recent efforts to extend our knowledge about stingless bee pollination. Additionally we discuss how future work on innate colour preference testing with stingless bees can proceed to help develop a more complete model of how these potentially important pollinators interact with flowers.

Materials and methods

Laboratory conditions for testing innate preferences

We tested the innate colour preferences of the Australian native stingless bee *T. carbonaria* that is a small black bee (1.13 ± 0.02 mm intertegulae span; mean \pm SD) that is amenable to experimental lab testing conditions (Spaethe et al. 2014). A photograph of this bee is shown in Spaethe et al. (2014); see Fig. 1b within that study. Experiments were conducted at Monash University in a 3 m \times 5 m controlled temperature laboratory (CT lab), set to 27 °C and 30 % relative humidity (SPER-Scientific Hygrometer, Arizona, USA) during habituation and experimental phases to allow effective foraging activity of the bees (Norgate et al. 2010). Two colonies of bees were propagated by Dr Tim Heard following established protocols (Heard 1988) and housed in 28 \times 20 \times 31 cm (LWH) pine nesting boxes. Each colony was connected to one of two identical foraging arenas by a 16 cm Plexiglas tube, which contained gates to control the movements of bees. The bees had no previous foraging experience, and were thus initially naïve with respect to flower stimuli. Pollen grains were provided directly to the nest box. It was possible to induce all forager bees to return to the colony by temporally lowering the ambient temperature in the CT lab to 19 °C (Norgate et al. 2010), which enabled easy control for cleaning equipment (10 % ethanol) and arranging stimuli for testing purposes.

Flight arena dimensions were 1.2 \times 0.6 \times 0.5 m (LWH) constructed with laminated white wooden side panels and a green plastic mat (Bunnings, VIC, Australia) floor. The arena lid was constructed of UV transparent Plexiglas as per Norgate et al. (2010). Illumination (10/14 h day/night) was provided by four Philips Master TLS HE slim-line 28 W/865 UV + daylight fluorescent tubes (Philips,

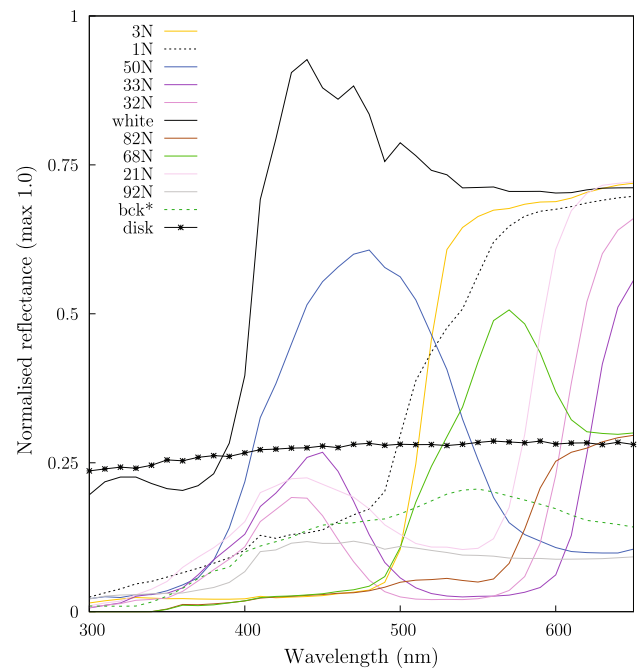


Fig. 1 Spectral reflectance of the 10 HKS colour stimuli, the green background (bck) and the aluminium disc priming stimuli. Normalised data plotted relative to a calibrated white BaSO₄ standard (Ocean Optics) that reflects radiation equally from 300 to 650 nm

Holland) with specially fitted high frequency (1200 Hz) ATEC Jupiter EGF PMD2614–35 electronic dimmable ballasts and diffused by Rosco 216 (Germany) UV transmitting screen (spectra shown in Farnier et al. 2015). This illumination approximately matches daylight illumination conditions for bees including the Australian context (Dyer 1998; Dyer and Chittka 2004).

The bees were initially allowed to habituate to the CT lab conditions for 7 days in which three plexiglas gravity feeders (von Frisch 1967) 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 lab, bee flight activity closely matched that of hives maintained outdoors (Heard and Hendrikz 1993; Norgate et al. 2010).

Stimuli and colorimetry

To measure the innate preferences of *T. carbonaria*, we used broadband HKS coloured cards as these stimuli have been used in several previous bee experiments (Giurfa et al. 1996; Dyer et al. 2008; Morawetz et al. 2013), and have spectral profile approximating the types of colours

that foraging bees might encounter in ecologically relevant scenarios (Chittka et al. 1994; Arnold et al. 2010). Artificial flower stimuli were cut in a circle (70 mm diameter) from standardised colour papers of the HKS-N-series (Hostmann-Steinberg K+E Druckfarben, H. Schmincke & Co., Germany). In each experiment, the same ten test colours (1N, pale yellow; 3N, saturated yellow; 21N, light pink; 32N, pink; 33N, purple; 50N, blue; 68N, green; 82N, brown; 92N, grey; back of 92N, white) were presented. Stimuli spectral characteristics including the green background were measured from 300 to 650 nm using an Ocean Optics spectrophotometer (S2000) coupled to a PX-2 pulsed xenon light source by a bifurcated, UV-vis, 600 μm probe. Data was processed using SPECTRA SUITE software (Ocean Optics Inc., Dunedin, FL, USA) and calibrated against a UV reflecting white BaSO_4 standard (Ocean Optics). To model the stimuli spectral characteristics (Fig. 1) considering hymenopteran vision, we used a hexagon colour space (Chittka 1992) which is generally applicable for hymenopteran trichromats, and has been used previously for mapping stingless bee colour vision (Spaethe et al. 2014). In model species like the honeybee, the analyses of colour choices modelled in either the Hexagon colour space or an independent receptor noise space (Kemp et al. 2015) are significantly correlated [Kendall's tau (τ) = 0.993, $P < 0.0001$] suggesting that colour choices are independent of specific model assumptions (Dyer and Neumeyer 2005; van der Kooij et al. 2016). We used spectral properties for the lab lighting (Farnier et al. 2015) and assumed that the colour visual system was adapted to the green background (Fig. 1).

As colour receptors are currently not known for *T. carbonaria*, we followed the principles outlined in (Kemp et al. 2015) and used spectral sensitivities for *Trigona spinipes* (Briscoe and Chittka 2001), the closest relative of *T. carbonaria* for which quality data exists. Spectral sensitivities were calculated using a vitamin A1 visual template assuming alpha-band peak sensitivities of 349, 445, 533 nm and a common beta band sensitivity of 340 nm (Stavenga et al. 1993). The hexagon model (Fig. 2) of bee vision calculates excitation values over the spectral range of 300–650 nm for the respective E(UV), E(blue) and E(green) photoreceptors of a bee and proposed subsequent neural processing mechanisms that facilitate colour perception (Chittka 1992).

We considered major factors that may contribute to 'colour' based foraging choices in bees. Specifically, we calculated (1) colour contrast as the Euclidean distance of a stimulus from the background (Chittka 1992), (2) dominant wavelength (hue) following established principles (Wyszecki and Stiles 1982; Chittka and Kevan 2005), (3) spectral purity (Wyszecki and Stiles 1982; Chittka and Kevan 2005), (4) brightness as the sum of E values (Spaethe et al. 2001), and (5) green contrast using the absolute

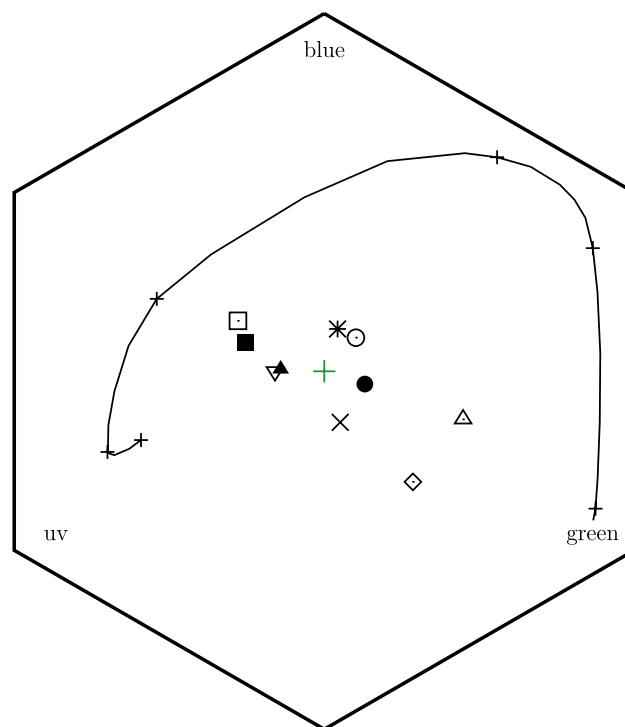


Fig. 2 Plots of HKS colour stimuli on hexagon space for *Tetragonula carbonaria* bee vision: 3N (diamond), 1N (multiplication sign), 50N (asterisk), 33N (open square), 32N (filled square), white (open circle), 82N (filled circle), 68N (open upward triangle), 21N (filled upward triangle) and 92N (downward triangle). Calculations were done assuming a chromatic adaptation to a green background. Refer to Table 1 for details on the chromatic properties of each target. Blue cross (+) indicates the locus of the green adaptation background. The curved line represents the spectral locus of theoretical pure spectral stimuli for *T. carbonaria* bees, and ticks on spectral locus indicate wavelengths from 350 to 550 nm at 50 nm intervals following methods in Chittka and Kevan (2005)

value of 0.5- E (green) as per Spaethe et al. (2001) since this receptor channel is implicated in being involved in driving behavioural responses in bees (Srinivasan and Lehrer 1988; Giurfa et al. 1996; Spaethe et al. 2001; Chittka and Kevan 2005).

Testing procedure

Following habituation two colonies were tested in identical arenas placed side-by-side, and testing order was pseudo-randomised with respect to each colony. Pilot tests established that bees did not land on colour stimuli unless pre-training was provided, which is consistent with previous innate preference testing in honeybees and bumblebees (Giurfa et al. 1995; Raine and Chittka 2007). Naïve *T. carbonaria* foragers 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), which produced a matt reflecting

Table 1 Visual characteristics of the colour targets used for the experiment

Stimuli	Colour category ^a	$E(U)$	$E(B)$	$E(G)$	Chromatic contrast	Brightness	Green contrast $(0.5 - E(G))$ ^b	Spectral purity (%)
1N	Green	0.653	0.536	0.704	0.149	1.89	0.204	27.2
3N	Green	0.410	0.244	0.696	0.396	1.35	0.196	46.0
21N	UV–blue	0.651	0.587	0.511	0.122	1.75	0.011	22.1
32N	UV–blue	0.529	0.483	0.275	0.235	1.29	0.225	32.9
33N	UV–blue	0.583	0.585	0.304	0.280	1.47	0.196	42.2
50N	Blue	0.642	0.782	0.684	0.124	2.11	0.184	17.1
68N	Green	0.138	0.228	0.586	0.410	0.952	0.086	66.3
82N	Green	0.148	0.179	0.279	0.119	0.606	0.221	29.8
92N	UV–blue	0.524	0.445	0.365	0.138	1.33	0.135	22.1
White	Blue–green	0.705	0.851	0.807	0.129	2.36	0.307	17.8

The different properties were calculated from reflectance spectra data following methods by (Chittka 1992; Chittka et al. 1994; Spaethe et al. 2001), and assuming a green background for chromatic adaptation (refer to “Materials and methods” for details)

^a Colour categories follow those by Chittka et al. (1994)

^b Senu (Spaethe et al. 2001)

surface. These stimuli equally reflect spectral radiation across the insect visual spectrum (Fig. 1). The disks were placed on vertical plastic cylinders of 100 mm height and 20 mm diameter. Bees were allowed a minimum of 2 h to forage on the pre-training disks, which were regularly replaced when sucrose was depleted. After pre-training, the temperature of the CT lab was lowered so that bees returned to the colony, and the arena could be cleaned.

The testing phase was conducted using the ten HKS colour stimuli (Table 1; Fig. 1) placed on the plastic cylinders. Stimuli were non-rewarded and presented at a random spatial position in the arena per trial. In a trial, ~40–50 bees were first isolated in the plexiglass tube, and then the gate to the arena was opened to allow the bees to start foraging. Four observers recorded choices by bees to stimuli over a 15 min period, where the number of landings on a colour was counted. A maximum of one landing (clear contact with a colour) was scored per approach to a stimulus by a bee. The 15 min testing was used based on pilot experiments showing that this period of time generated sufficient numbers of choices by stingless bees to potentially dissect innate preference factors, whilst limiting choices to an initial period to be consistent with innate testing principles (Giurfa et al. 1995; Raine and Chittka 2007). The frequency of bees choosing a stimulus was low, in the range of about one choice every 10–20 s during the tests. Individual bees tended to only land on a colour for a short period (1–3 s) before taking flight again. 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 was independent and driven by individual colour visual choices rather than social cues. Whilst it is difficult to precisely track unmarked bees, the

observation of data recorders was that a majority of bees were involved in active flight and making colour choices. The experiment continued for 45 min to allow for the collection of additional data not pertinent to the innate preference questions under consideration in the current study. At the completion of a trial, the bees were sacrificed so data were independent to avoid pseudo replication. From each colony, six replicates were conducted with fresh stimuli, and testing order between colonies in the respective arenas was pseudo-randomised.

Statistical analysis

Results from the behavioural experiment were first analysed by means of a contingency table with two factors: colony (two levels) and colour category (ten levels). The number of landings on each colour target during the first 15 min of the experiment was used as the dependent variable. We tested for null hypothesis of independence between the two factors by means of a Pearson's Chi-squared test, and analysed the standardised residuals to identify potentially significant associations between the different colour stimuli and the frequency of landings. The statistical analysis was performed using the routine Cross Tabs available for the R statistical programming language 3.2.3 (R Core Team 2015).

Subsequent analyses considered the effect of visual appearance of colour targets on innate preferences. For this purpose, a multiple regression analysis was performed to understand the potential effect of four previously identified spectral characteristics of the stimuli: (1) brightness, (2) green contrast, (3) chromatic contrast and (4) spectral purity on the number of landings observed for each colour (Wyszecki and Stiles 1982; Giurfa et al. 1996, 1997; Spaethe et al. 2001; Kemp et al. 2015).

The regression analysis was done using a count regression model (a case of the generalised linear model) assuming a Poisson distribution and a logit link to account for the discrete nature of the response variable (Faraway 2006). Analyses were done using the glm routine available for the R statistical programming language 3.2.3 (R Core Team 2015).

Results

Innate colour preference by *T. carbonaria*

Statistical analysis of innate colour preferences for the two colonies of *T. carbonaria* revealed a significant interaction between colony and frequency of landings on the different colour stimuli [$\chi^2(9) = 30.8, p \text{ value} = 0.0003$]. This result suggests the existence of differences in the innate preference for colour between the two colonies.

Analysis of the individual standardised residuals revealed that only bees corresponding to colony 1 presented a significant deviation from the expected frequency of visits for each colour (Table 2). Moreover, individuals from colony 1 showed a significantly higher number of visits to the both the 'white' and 'blue' (HKS50N) colour stimuli, and a significantly lower number of visits to the 'green' (HKS68N) colour stimulus than those expected by chance (Table 2).

Effects of visual appearance

An initial correlation analysis was performed on four predictor variables: brightness, green contrast, chromatic contrast and spectral purity to reduce potential effects of multicollinearity in the regression model. This analysis revealed a significant correlation between brightness and spectral purity variables [Spearman's rank

correlation (r_s) = $-0.669, P \text{ value} = 0.034$], and between spectral purity and chromatic contrast ($r_s = 0.766, P \text{ value} = 0.010$). For subsequent analyses it was important to reduce factors based upon the biological plausibility of cause. Studies on honeybee vision suggest brightness is not a major perceptual mechanism when colour is being processed (Backhaus et al. 1987; Backhaus 1991; Reser et al. 2012), and since this factor correlates with purity, it is reasonable to remove this potential factor from the model whilst retaining purity. Furthermore, studies on bumblebee (Lunau 1990; Lunau et al. 1996; Rohde et al. 2013) and honeybee (Rohde et al. 2013) perception show that for similar colours, purity is an honest signal that some bee pollinators do show an innate preference towards; therefore, it is biologically relevant to retain this factor and remove the correlating factor of chromatic contrast from the model.

Regression analyses suggested that green contrast and the interaction between this factor and spectral purity have a significant effect on the number of observed landings in naïve *T. carbonaria* bees [Deviance (G) green contrast = 22.7, $P \text{ value} = 0.004$; Ggreen contrast \times purity = 8.95, $P \text{ value} = 0.023$]. Interestingly, the factor of purity by itself was not found to have a significant effect on the number of landings (Gpurity = 0.23634, $P \text{ value} = 0.768$). Individual plots for the main effects and the interaction term are depicted in Fig. 3.

Effects of dominant wavelength

Another potential factor of colour perception is hue, which can be described by the dominant wavelength of a stimulus (Wyszecki and Stiles 1982; Chittka and Kevan 2005; Kemp et al. 2015). This potential factor was considered separately (Fig. 4) since previous work on honeybees shows that the effect of hue on choices cannot be explained with

Table 2 Contingency table displaying the cross-classification of the number of landings for ten different colours observed for *Tetragonula carbonaria* bees belonging to two different colonies

	1N	3N	21N	32N	33N	50N	68N	82N	92N	White	Key
Colony 1	15	14	11	28	24	29	20	17	15	50	I
	20.2	19.5	16.0	21.2	20.5	20.2	31.4	17.4	19.1	37.6	II
	6.73 %	6.28 %	4.93 %	12.6 %	10.8 %	13.0 %	8.97 %	7.62 %	6.73 %	22.4 %	III
	-1.15	-1.24	-1.26	1.48	0.780	1.97	-2.04	-0.100	-0.940	2.03	IV
	0.250	0.215	0.208	0.139	0.435	0.049*	0.041*	0.920	0.347	0.042*	V
Colony 2	44	43	36	34	36	30	72	34	41	60	I
	38.9	37.5	31.0	40.8	39.5	38.9	60.6	33.6	36.9	72.4	II
	10.2 %	10.0 %	8.37 %	7.91 %	8.37 %	6.98 %	16.7 %	7.91 %	9.53 %	14.0 %	III
	0.830	0.890	0.910	-1.07	-0.560	-1.42	1.47	0.070	0.680	-1.46	IV
	0.407	0.373	0.363	0.285	0.576	0.156	0.142	0.944	0.497	0.144	V

For each colony, rows represent: observed number of landings (I), expected number of landings (II), percentage of landings per colour target (III), standardised residuals (z scores) (IV) and their corresponding probability (two-tailed) (V)

* Indicates significant values at $\alpha = 0.05$

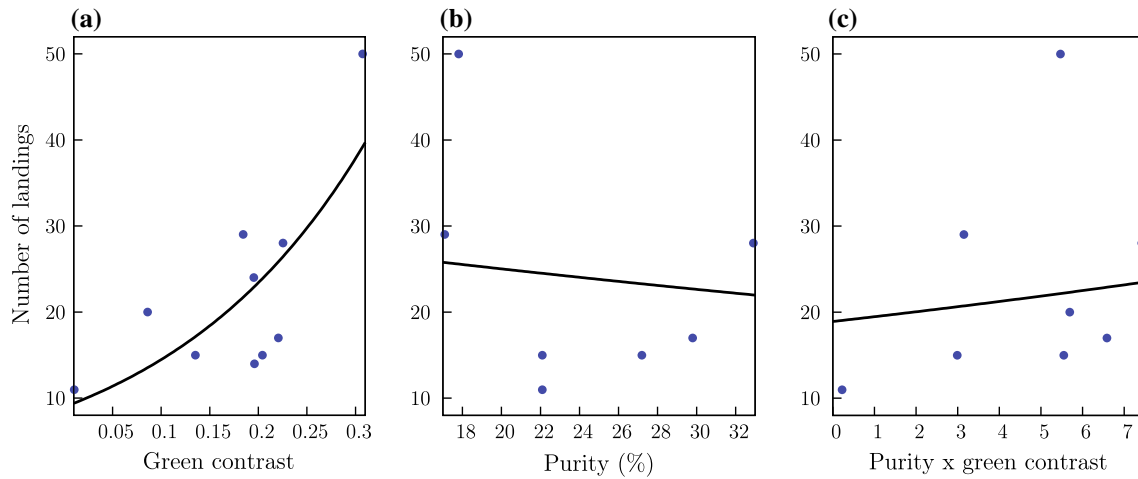


Fig. 3 Analyses of innate colour preference choices for colony 1 of the stingless bee *T. carbonaria*. Regression models (*solid lines*) for the main effects and interaction term of the regression model explaining the relationship between number of observed landings (y axis) and various colour characteristics of the colour targets: (a)

green contrast, (b) spectral purity and (c) interaction term (green contrast \times purity). Green contrast and the interaction term were both found to have a significant effect on the number of landings observed at $\alpha = 0.05$, whilst purity was not significant as a sole factor (see text for full statistics)

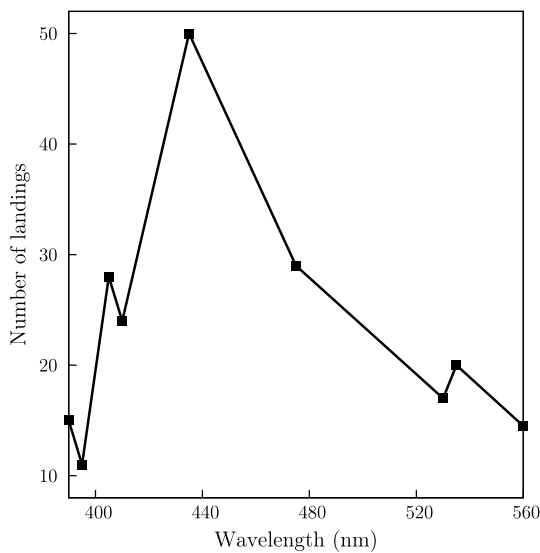


Fig. 4 Number of choices by stingless bee *T. carbonaria* (colony one) plotted against the dominant wavelength corresponding to each of the *different colour* targets used for the experiment. Dominant wavelength values were obtained assuming chromatic adaptation to a *green background* as per Chittka and Kevan (2005)

conventional regression analyses (Menzel 1967; Giurfa et al. 1995).

Figure 4 shows that there appears to be an effect of rich ‘blue’ wavelengths on the colour preference by *T. carbonaria*. While, the current study employed broadband stimuli and was not specifically designed to dissect the potential spectral preferences for specific wavelengths at high resolution, the results do bear a resemblance to previous reports for honeybees (Menzel 1967; Giurfa et al. 1995).

Discussion

Colour preferences in flower visiting insects are a plausible way that naïve individuals are able to first find flowers to collect nutritional rewards (Giurfa et al. 1995; Raine and Chittka 2007). Interestingly, work on bumblebee innate preferences have shown that inter colony variability exists and colour preferences may help colonies forage more efficiently from profitable flowers (Raine and Chittka 2007). In the current study, we also observed a significant difference between the innate colour choices of *T. carbonaria* bees where colony 2 had choices that were not significantly different from chance expectation, whilst colony 1 did show significant preferences towards certain stimuli.

The selected broadband stimuli covered a range of possible colours that might occur in natural settings (Figs. 1, 2). Bee choices correlated with green contrast and also showed a significant interaction effect between green contrast and spectral purity. However, spectral purity as a sole factor did not correlate with *T. carbonaria* choices, which is in contrast with recent work on honeybees and bumblebees where there was a preference of similar colours of higher spectral purity compared to trained colours (Papiorek et al. 2013; Rohde et al. 2013). A possible explanation for this difference is that in the current study, the colour stimuli were well spread in colour space and dissimilar from each other (Fig. 2). As such, it is possible that a variety of physiological factors that contribute to colour perception (Wyszecki and Stiles 1982; Giurfa et al. 1996, 1997; Spaethe et al. 2001; Kemp et al. 2015) may interplay to drive innate colour responses. In the Rohde et al. (2013) study, the stimuli

were very similar and from a confined region of bee colour space, and it has been well-established that colour processing in bees operates in different ways for either similar, or dissimilar colours (Dyer and Chittka 2004). Given the evidence that *T. carbonaria* does exhibit innate preferences that show a significant correlation between factors of green contrast and spectral purity, this suggests it will be of value to further consider spectral purity of colour signals in stingless bees using similar colour stimuli. For example, another recent study on the stingless bee species *Melipona mondury* and *Melipona quadrifasciata* (Koethe et al. 2016 this issue) from Brazil suggests a complex interaction between different colour parameters of stimuli and the preference choices in experienced foraging bees. These new studies thus suggest that it will be of high value to test additional bee species in different regions and foraging conditions, to better understand how colour preferences may influence flower choices for ecological and agricultural purposes.

The evidence of a green contrast factor in *T. carbonaria* innate choices is unexpected; but interestingly, honeybees in addition to their preference for blue colour stimuli do also show a weak preference for longer wavelength radiations (Giurfa et al. 1995). In several bee species, green contrast improves the capacity to detect stimuli when combined with chromatic contrast (Giurfa et al. 1996; Spaethe et al. 2001; Morawetz et al. 2013), although this may not be true for stimuli lacking green contrast (Giurfa et al. 1996). This suggests that bee behavioural responses to the various factors used to quantify colour can often not be explained in a straightforward way (Kemp et al. 2015).

Another interesting component of our analyses was the consideration of dominant wavelength (Fig. 4) for the stimuli defined by colour properties in Table 1. *Tetragonula carbonaria* bees showed a significant preference for the 'white' stimulus with dominant wavelength of about 435 nm and the 'blue' HKS50N stimulus with dominant wavelength of about 475 nm, which mirrors the findings on honeybees preferring stimuli with dominant wavelength in the blue region of the spectrum (Menzel 1967; Giurfa et al. 1995). However, there is an important consideration when comparing innate responses to very narrow-banded, quasi-monochromatic stimuli to responses induced by broadband stimuli as those commonly observed in flowers (Chittka et al. 1994; Arnold et al. 2010). In fact, one type of colour photoreceptor can be excited by different combinations of wavelength and intensity, and the brain cannot perceive a difference as shown by the principle of univariance (Rushton 1972; Garcia et al. 2015). This means that one photoreceptor type can therefore not differentiate between changes in wavelength and changes in intensity within the spectral range of that photoreceptor; thus, innate responses to narrow and broadband stimuli cannot be directly compared with each other in a straight forward way. Therefore,

when considering an ecologically meaningful case of broadband stimuli and potential pollinator preferences, the use of colour categories employing a biologically relevant bee colour space (Chittka et al. 1994; Raine and Chittka 2007) is a realistic assumption to map the range of stimuli encountered in complex natural conditions.

Using the categorical definitions of Chittka et al. (1994), *T. carbonaria* bees showed a significant preference for stimuli from the blue and blue-green regions of the colour hexagon, consistent with findings that honeybees and bumblebees tend to prefer flowers with such spectral characteristics (Giurfa et al. 1995; Raine and Chittka 2007). While it remains to be definitively shown whether bee innate colour preferences may drive flower evolution, there is evidence that such preferences are linked to flowers of these hues having higher nectar rewards (Giurfa et al. 1995; Raine and Chittka 2007). Here, an outstanding question is, whether bees evolved innate preferences because certain flower types are more rewarding (Raine and Chittka 2007) and/or whether flowers having such hues subsequently receive sufficient fitness benefit so as to evolve the capacity to better offer higher rewards because the hues are linked to an underlying physiological mechanism of bee colour processing (Chittka and Wells 2004). This question presents interesting experimental possibilities to test if flowers from the blue region of colour space in Australia also present higher rewards to stingless bee pollinators. To undertake such research, it is important to understand the extent to which flower coloration in an environment might be limited by biochemical or phylogenetic constraints as suggested by some authors (Menzel and Shmida 1993), or if flower colour is plastic as recently demonstrated using phylogenetically informed statistical analyses of flower coloration from the sub-tropical or sub-alpine regions of the Nepalese Himalayas (Shrestha et al. 2014).

In Australia, there have been recent advancements in the capacity to map flower colouration as perceived by pollinators using phylogenetically informed analyses (Dyer et al. 2012; Shrestha et al. 2013) which reveals that flower colour in Australia is indeed very plastic between different plant families. This result strongly suggests that plant flowers could evolve blue flowers with high rewards if this promoted sufficient fitness benefits by attracting native pollinators with blue preferences. Thus, the future comparison of Northern Hemisphere and Australian data sets for innate preferences, flower colouration and nectar volume should enable a capacity to compare the extent to which innate preferences do influence the types of flower colours that evolve in different conditions.

In recent times, there has been a growing appreciation that stingless bees are important pollinators of potential high value for both ecological and agricultural purposes (Heard 1994, 1999; Dollin et al. 1997; Heard and Dollin 1998; Dollin 2010; Halcroft et al. 2013). Honeybees and bumblebees have

been well-established models of pollination, partially due to the experimental access available from these species for the collection of high quality data. With stingless bees like *T. carbonaria* and *Trigona cf. fuscipennis*, a stingless bee species from Costa Rica; it has been necessary to develop protocols for mass training and testing bees because individuals appear reluctant to forage in isolation. This does present some statistical challenges because of the need to collect sufficient data choices to enable a robust comparison, whilst maintaining independence of data. We were able in the current experiment to strike a balance by testing small groups of *T. carbonaria* on multiple tests, where bees from a given group were sacrificed at the completion of their test. In addition, during our pilot experiments, there was a high mortality rate if bees were marked using standard methods for marking honeybees as in (von Frisch 1967). In spite of these challenges, it was possible to collect data from stingless bees in the current study to test *T. carbonaria* innate preferences to understand how these pollinators may interact with flowers in the Australian context.

The similar distribution of flower marker points from the Northern and Southern hemisphere (Chittka and Menzel 1992; Dyer et al. 2012; Shrestha et al. 2013) fit with ideas of similar levels of selective pressure following visual ecology principles (Lythgoe 1979). Future work could consider how the colour preferences of *T. carbonaria* may influence their interaction with high value native flowers, how bee colour preferences may be affected by the type of natural background context where flowers exist (Neumeyer 1980; Giurfa et al. 1995), and how the potential factor of saturation may influence decision making for similarly coloured stimuli (Rohde et al. 2013).

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Compliance with ethical standards

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

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