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Impact of colour preferences on the foraging behaviour of tropical stingless bees

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ABSTRACT

Social bees are known as important pollinators in their respective habitats, however, concerning many aspects of plant-pollinator interactions single species such as *Apis mellifera* or *Bombus terrestris* are investigated and general conclusions are drawn for social bees. Stingless bees are by far the most speciose and diverse tribe of social bees, with more than 500 described species. In comparison to the domesticated Western honey bee, stingless bees produce less honey, but they have proven to be effective pollinators for many crops, including unsuitable crops for honey bees like buzz-pollinated plants in greenhouses. Stingless bees are distributed throughout the Tropics, where honey bees are introduced for pollination services of crops and the production of honey. Honey bees are not the best pollinators for many crop plants, but their easy handling, colony size, honey production and the vast knowledge that was gathered concerning their biology over the past centuries make them important livestock for humans.

In this study, we investigated colour preferences of stingless bees to analyse whether all social bees prefer similar colours or if their choice behaviour is divergent. Previous studies investigated the close association between floral colour reflectance and bee colour perception. Based on these information, we assumed that colour perception in bees should be similar in means of preferences for specific colour traits of flowers, like bluish hues and high values of saturation because both parameters can be associated with higher nectar rewards.

First, innate preferences of the Australian native stingless bee *Tetragonula carbonaria* were analysed by offering small groups of foragers a choice between ten differently coloured broadband stimuli under laboratory conditions. The workers chose bluish colours more often and were influenced by higher green contrast and an interaction between green contrast and saturation. In an additional reciprocal experiment with *T. carbonaria*, experienced workers were individually trained to forage in an arena. They were given the choice between stimuli of the same blue colour hue that either differed in saturation or brightness. Neither brightness nor saturation influenced their colour choices. The same experiment was conducted with *A. mellifera* that chose high saturated colours significantly more frequent, while showing no preferences based on brightness.

Two experiments concerning colour preferences of stingless bees were executed in Brazil. In the first experiment, workers of two *Melipona* species had to make quadruple choices of different colour hues or of the same colour hue with different variations of the colour parameters brightness and saturation. *M. quadrifasciata* preferred yellow, whereas *M. mondury* preferred UV-reflecting blue. In the second experiment, *M. bicolor* and *Partamona helleri* were individually trained and had to make 57 dual choices, in which colours with differing hues or differing values of brightness and saturation were offered. While *M. bicolor* generalized all colours, workers of *P. helleri* chose bluish colour hues significantly more frequent and also preferred high values of saturation. The results obtained for *P. helleri* refer to known preferences of honey bees and bumble bees, while the results for the other tested stingless bee species do not resemble those of other social bees.

One reason for the divergence of colour preferences between most stingless bees and honey bees could be the communication via olfactory signals by stingless bees, so called scent marks. Stingless bees are known to use scent marks for the recruitment of nestmates. The last experiment of this study explored the importance of deposited scent marks, colour and location on the bees' selection of food sources. *M. subnitida* and *Plebeia flavocincta* were trained to forage on mass feeders in Brazil, whereas honey bees were trained the same way in Germany. All three species significantly preferred the feeder that was previously scent marked by conspecifics. When given the choice between a blue and a yellow feeder honey bees chose the blue feeder with strong fidelity, while the stingless bees preferred the feeder they were trained to in most cases. *M. subnitida* additionally preferred feeders with closer proximity to their hive.

In total, the results of this study suggest that colour preferences in social bees have some similarities, but overall colour seems to be less important to stingless bees. The recruitment via scent marks or adaptations to their respective habitat could be responsible for the differences of colour preferences in social bees. Ambient light conditions as well as physiological characteristics, like the size of the bees' eyes that correlate with body size which is smaller in many stingless bees compared to honey bees, could be reasons for poorer colour vision or a weaker dependence on visual cues.

ZUSAMMENFASSUNG

Soziale Bienen sind als wichtige Bestäuber in ihren jeweiligen Lebensräumen bekannt, allerdings werden hinsichtlich vieler Aspekte der Wechselwirkungen zwischen Pflanzen und Bestäubern einzelne Arten wie *Apis mellifera* oder *Bombus terrestris* untersucht und verallgemeinerte Schlussfolgerungen für soziale Bienen gezogen. Stachellose Bienen sind mit Abstand der artenreichste und vielfältigste Tribus sozialer Bienen, mit mehr als 500 beschriebenen Arten. Im Vergleich zu der domestizierten Westlichen Honigbiene produzieren stachellose Bienen weniger Honig, aber sie haben sich als effektive Bestäuber für viele Nutzpflanzen erwiesen, auch für Nutzpflanzen, die für Honigbienen ungeeignet sind, wie beispielsweise für vibrationsbestäubte Pflanzen in Gewächshäusern. Stachellose Bienen sind in den Tropen verbreitet, wo Honigbienen für die Bestäubung von Nutzpflanzen eingeführt werden. Honigbienen sind nicht die besten Bestäuber für viele Nutzpflanzen, aber ihre einfache Handhabung, Koloniegröße, Honigproduktion und das große Wissen über deren Biologie, das in den letzten Jahrhunderten gesammelt wurde, machen sie zu wichtigen Nutztieren für den Menschen.

In dieser Studie untersuchten wir die Farbpräferenzen stachelloser Bienen, um zu analysieren, ob alle sozialen Bienen ähnliche Farben bevorzugen oder ob ihr Auswahlverhalten unterschiedlich ist. Frühere Studien untersuchten den engen Zusammenhang zwischen floraler Farbreflexion und der Farbenwahrnehmung von Hummeln und Honigbienen. Basierend auf diesen Informationen gingen wir davon aus, dass die Farbwahrnehmung verschiedener Bienenarten ähnlich sein sollte, was die Präferenzen für bestimmte Farbeigenschaften von Blüten betrifft, wie bläuliche Farbtöne und hohe Sättigungswerte, da beide Parameter mit höheren Nektarbelohnungen verbunden sein können.

Zunächst wurden die angeborenen Präferenzen der australischen stachellosen Biene *Tetragonula carbonaria* analysiert, indem kleinen Gruppen von Sammlerinnen die Wahl zwischen zehn verschiedenfarbigen Breitbandstimuli unter Laborbedingungen geboten wurde. Die Arbeiterinnen wählten häufiger bläuliche Farben und wurden durch Grünkontrast und einem Zusammenspiel von Grünkontrast und Sättigung beeinflusst. Darauf folgend wurde ein reziproker Wahlversuch mit *T. carbonaria* durchgeführt, in welchem erfahrene Arbeiterinnen individuell für die Futtersuche in einer Arena trainiert wurden. Die Arbeiterinnen

hatten die Wahl zwischen Stimuli mit dem gleichen Blauton, die sich entweder in ihrer Sättigung oder in ihrer Helligkeit unterschieden. Weder Helligkeit noch Sättigung beeinflussten die Farbwahl der Arbeiterinnen. Das gleiche Experiment wurde mit *A. mellifera* durchgeführt, die deutlich häufiger Farben mit hoher Sättigung wählten, ohne Präferenzen aufgrund der Helligkeit zu zeigen.

Zwei Experimente zur Farbbevorzugung von stachellosen Bienen wurden in Brasilien durchgeführt. Im ersten Experiment mussten die Arbeiter zweier *Melipona*-Arten eine Wahl aus vier verschiedenen Farbtönen oder demselben Farbton mit je vier unterschiedlichen Variationen der Farbparameter Helligkeit und Sättigung treffen. *M. quadrifasciata* bevorzugte gelb, während *M. mondury* UV-reflektierendes blau bevorzugte. Im zweiten Experiment wurden *M. bicolor* und *Partamona helleri* individuell trainiert und mussten 57 mal aus je zwei Stimuli wählen, in denen Farben mit unterschiedlichen Farbtönen oder unterschiedlichen Helligkeits- und Sättigungswerten angeboten wurden. Während *M. bicolor* alle Farben generalisiert, wählten die Arbeiterinnen von *P. helleri* deutlich häufiger bläuliche Farbtöne und bevorzugten hohe Sättigung. Die für *P. helleri* erzielten Ergebnisse entsprechen bekannten Präferenzen von Honigbienen und Hummeln, während die Ergebnisse der anderen getesteten stachellosen Bienenarten nicht denen anderer sozialer Bienen entsprechen.

Ein Grund für die Divergenz der Farbpräferenzen zwischen den meisten stachellosen Bienen und Honigbienen könnte die Kommunikation über Duftsignale von stachellosen Bienen, sogenannte Duftmarken, sein. Es ist bekannt, dass stachellose Bienen Duftmarken für die Rekrutierung von Mitgliedern ihres Nests verwenden. Das letzte Experiment dieser Studie untersuchte die Bedeutung der gesetzten Duftmarken, der Farbe und der Lage auf die Auswahl der Nahrungsquellen der Bienen. *M. subnitida* und *Plebeia flavocincta* wurden in Brasilien trainiert, an Zuckerwasserspendern zu furagieren, während Honigbienen in Deutschland auf die gleiche Weise trainiert wurden. Alle drei Arten bevorzugten signifikant die Futterspender, die zuvor von Artgenossen markierten wurden. Bei der Wahl zwischen einem blauen und einem gelben Futterspender wählten die Honigbienen den blauen Futterspender am häufigsten, während die stachellosen Bienen die zuvor trainierte Farbe bevorzugten. Allein *M. subnitida* bevorzugte zusätzlich Futterspender, die näher an ihrem Nest positioniert waren.

Insgesamt deuten die Ergebnisse dieser Studie darauf hin, dass die Farbpräferenzen bei sozialen Bienen einige Ähnlichkeiten aufweisen, aber insgesamt scheint Farbe für stachellose Bienen für die Blütenwahl weniger wichtig zu sein. Die Rekrutierung über Duftmarken oder Anpassungen an den jeweiligen Lebensraum könnte für die unterschiedlichen Farbpräferenzen der sozialen Bienen verantwortlich sein. Umgebungslichtverhältnisse sowie physiologische Eigenschaften, wie die Größe der Augen, die mit der Körpergröße korrelieren, die bei vielen stachellosen Bienen im Vergleich zu Honigbienen kleiner ist, können Gründe für ein schlechteres Farbsehen oder eine schwächere Abhängigkeit von visuellen Signalen sein.

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CHAPTER 1

General Introduction

Stingless bees – ecological and behavioural aspects

The distribution of stingless bees (Meliponini) is widely spread and ranges throughout the Pantropics (Indo-Australia, Neotropics, Africa-Madagascar), including continental disjunctions (Fig. 1A; Michener 2007; Rasmussen 2017). With more than 500 species stingless bees are the most speciose bee tribe (Michener 2013). Environments that are inhabited by stingless bees are very diverse and range from sparsely vegetated areas to densely vegetated forests (Heard 2016). Based on the richness of species and their wide-spread habitats, a variety of characteristics developed in stingless bees, which makes them a more diverse tribe than other Apidae, e. g. honey bees and bumble bees (Michener 2007; Hrncir & Maia-Silva 2013). The most striking similarity between stingless bees, honey bees and bumble bees is their eusocial lifestyle. Honey bees use their waggle dance to share information and their division of labour facilitates the organization as a superorganism (von Frisch 1967; Seeley 1989). Bumble bees and stingless bees on the other hand use less elaborated communication techniques, like vibrations and excited movements

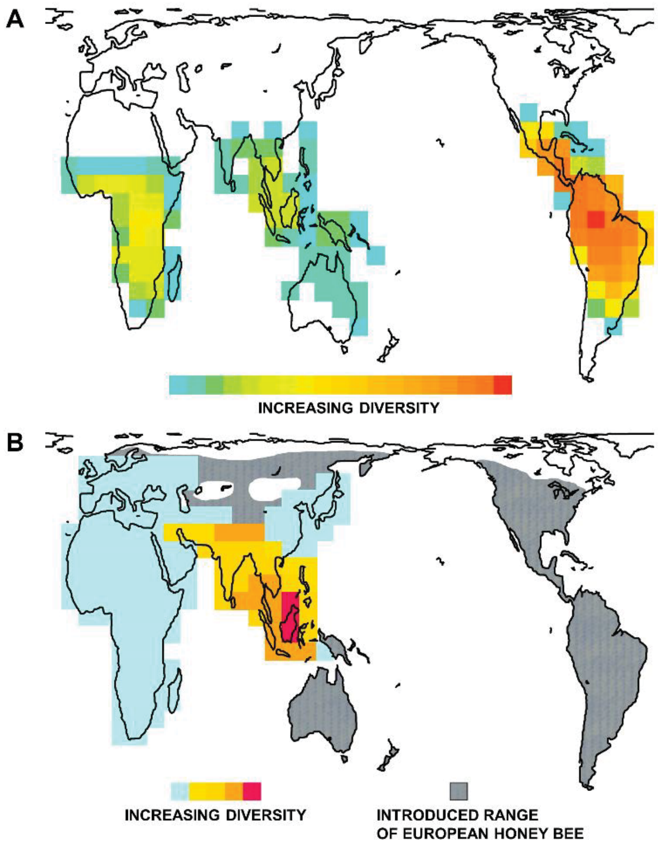

Fig. 1 Distribution of stingless bees (A) and honey bees (B). The colour scale represents the abundance of species with pale blue indicating few and red indicating high numbers of species. (Figures from Heard 2016)

(Lindauer & Kerr 1960; Nieh 2004). Another way of communication that is used by eusocial bees are scent marks (Stout & Goulson 2001; Hrncir et al. 2004; Gawleta et al. 2005; Wilms & Eltz 2008). Honey bees and bumble bees use so called olfactory footprints that are involuntarily deposited scent marks to recognize and avoid previously depleted flowers, but are also able to actively deposit attractive or repellent scent marks (Free & Williams 1983; Corbet et al. 1984; Giurfa & Nunez 1992; Giurfa 1993; Stout et al. 1998; Stout & Goulson 2001; Schmidt et al. 2003). Stingless bees have a more elaborated communication via scent marks (Lindauer & Kerr 1960; Schmidt et al 2003; Hrncir et al. 2004; Jarau et al. 2004). Different species use varying techniques like the deposition of scent marks on a food source itself or scent trails that lead workers from the hive to a food source, and the discussed scent tunnels that allow bees in densely vegetated forests, where wind is not so prevalent, to fly through a tunnel of scent molecules to find the location of rewarding food sources (Kerr 1969, 1994; reviewed by Nieh 2004). These scent marks are nest specific, but bees of different hives and species are able to recognize scent marks of others and can adopt their behaviour by following the scent marks or by avoiding them (Barth et al 2008; Jarau 2009; Lichtenberg et al. 2011). Within the tribe of stingless bees, characteristics like the arrangement of brood cells, queen production, foraging strategies, nesting biology, colony size and body size differ between species (Michener 1974; Johnson 1983; Wille 1983; Engels & Imperatriz-Fonseca 1990; Roubik 2006, Barth et al. 2008).

During foraging, workers of stingless bees often appear in groups and they can be divided into two categories: aggressive and non-aggressive foragers (reviewed by Biesmeijer & Slaa 2004). Both groups try to collect rewards efficiently by exploiting these in large numbers. So called scout bees are searching for rewards and recruit other workers (Biesmeijer & Slaa 2004). By using additional food sources close to the originally found food source non-aggressive foragers try to exploit rich and clumped food sources rapidly (Nagamitsu et al. 1999; Slaa 2003). Aggressive foragers on the other hand not only rely on scout bees to find new rewards, but also to detect other bees while foraging and then recruit nestmates to the occupied reward to take it over (Biesmeijer & Slaa 2004). Non-aggressive foragers like *Melipona* and *Partamona* species have been observed sharing food sources with honey bees, while aggressive foragers like *Trigona* species avoid to share food sources with honey bees (Roubik 1980). Aggressive foragers also appear to be more generalistic in their choice of food plants than non-aggressive foragers (Roubik 1978; Ramalho 1990).

Honey bees are valued by humans because of their pollination services and their production of honey and, therefore, had been introduced worldwide (Fig. 1B). But also stingless bees have proven to be efficient pollinators of many crop plants and some species produce honey (Heard 1994, 1999; Kremen et al. 2004; Souza et al. 2006; Rodríguez-Malaver et al. 2009). The growing human population demands increased food production that is hard to achieve solely with honey bees that struggle in consequence of colony collapse disorder, diseases, parasites, habitat loss and effects of intensified agricultural land use (e. g. pesticides). Many stingless bee species are able to substitute the services of honey bees, but are less popular because of smaller colony sizes, less available information and their reduced honey production. Nonetheless, stingless bees also have advantageous traits, like their missing sting that makes them less harmful especially in urban areas and many species are able to buzz-pollinate crops like tomato or eggplant, an ability honey bees do not possess (Del Sarto et al. 2005; Dos Santos et al. 2009; Nunes-Silva et al. 2013). Introduced honey bees are discussed to be a threat to native bee species and to influence the diversity of communities (Roubik 1978, 1989; Roubik et al. 1986; Thomson 2004). By involving stingless bees in the pollination of crop plants, the introduction of honey bees to non-native habitats can be reduced or prevented.

Properties of colour

Colour is defined as the reflected light of an object that is subjectively perceived by an organism via its photoreceptors. The received light waves are transformed into specific signals by different receptor types that are stimulated by specific wavelengths. For example, humans are trichromatic (Fig. 2A) with photoreceptors most sensitive in the blue, green and red waveband (Bowmaker & Dartnall 1980). Besides the three photoreceptor types that enable colour discrimination in bright light conditions, also called cones, humans have another type of photoreceptor for dim-light conditions that are more sensitive than cones, called rod cells, that are mainly used to discriminate brightness contrasts under dim-light conditions (Baylor et al. 1979, 1984). In the visible spectrum of humans (390 – 700 nm), blue has the shortest and red the longest wavelength. Each type of photoreceptors follows the principle of univariance, which means that a single type of photoreceptor is not able to cause colour perception but that stimulations of different photoreceptor types are calibrated against each other by the brain of the perceiver to create the impression of colour (Rushton 1972; Hagendorf 2011; Garcia et al. 2015). To understand how colours are perceived three levels need to be taken into consideration – the physical, physiological and psychological level (Bachmann & Bernhard 2011). The physical level is the electromagnetic radiation, the light impulse that meets the eye of the beholder. There are two different possibilities how a light impulse can reach the eye. First, the light meets the eye directly e. g. the sunlight when you look at the sun or second, the light meets an object that reflects the light and meets the eye as the reflected light. If the light meets the eye directly solely the characteristics of the light is taken into consideration, but if the light is reflected by an object the characteristics of the surface manipulate the light that is received by the eye, e. g. sunlight that is reflected by the surface of the moon. The physiological level is based on the colour stimulus specification and describes the processes within the eye when light is perceived. Light that is perceived by the eye stimulates the different photoreceptor types. In case of trichromatic organisms, e. g. humans and bees, the excitation of the three different photoreceptor types generate the colour stimulus specification (Böhringer et al. 2011). The third level is the sensation of colour and describes the translation of the collected physiological information to neurological information that is processed in the brain. At the physical level colour stimuli are described by the spectral composition, resp. reflection, at the physiological level by its intensity, dominant wavelength and colour purity and at the level of colour perception by its brightness, hue and saturation.

Colour is a subjective impression and cannot be quantified (Richter 1981). Nonetheless, it is generally accepted to describe colour in terms of hue, saturation and brightness (Bachmann & Bernhard 2011). The hue of a colour describes the perceived shade (blue, green, yellow, red) on the psychological level, but can also be characterized by the monochromatic light that is predominantly perceived by the photoreceptors (physiological level) or solely reflected from an object (physical level). Saturation of a colour can be described as the distribution of reflectance across the spectrum. A narrow range of reflected wavelengths causes high saturation, while a broad range of reflected wavelengths causes low saturation. Achromatic colours reflect all or none wavelengths in the visible range of the beholder and, therefore, are unsaturated. Psychologically, saturation is the degree of colourfulness that the observer perceives. Lastly, brightness is the total amount of reflected light that is perceived by the photoreceptors. On the psychological level brightness describes the perceived lightness or darkness of a colour and physically it is the quantitative amount of light that is reflected from an object.

Colour vision of bees

The capability of bees to perceive and discriminate colours has been studied for more than a century (Dyer & Arikawa 2014, Dyer et al. 2015). Nobel Prize laureate Karl von Frisch was the first to demonstrate colour vision in Western honey bees (*Apis mellifera*) by training individual workers to a blue-coloured cardboard. In the subsequent test these workers were offered a choice between different shades of grey – of which some had the same brightness as the previously trained blue cardboard – and the known blue cardboard (von Frisch 1914, 1967). The position of the blue stimulus was randomly chosen, but the workers remained to choose the blue cardboard, although it offered no reward anymore. In the past decades, new technologies allowed scientists to analyse the visual system of a variety of organisms in more detail, including the

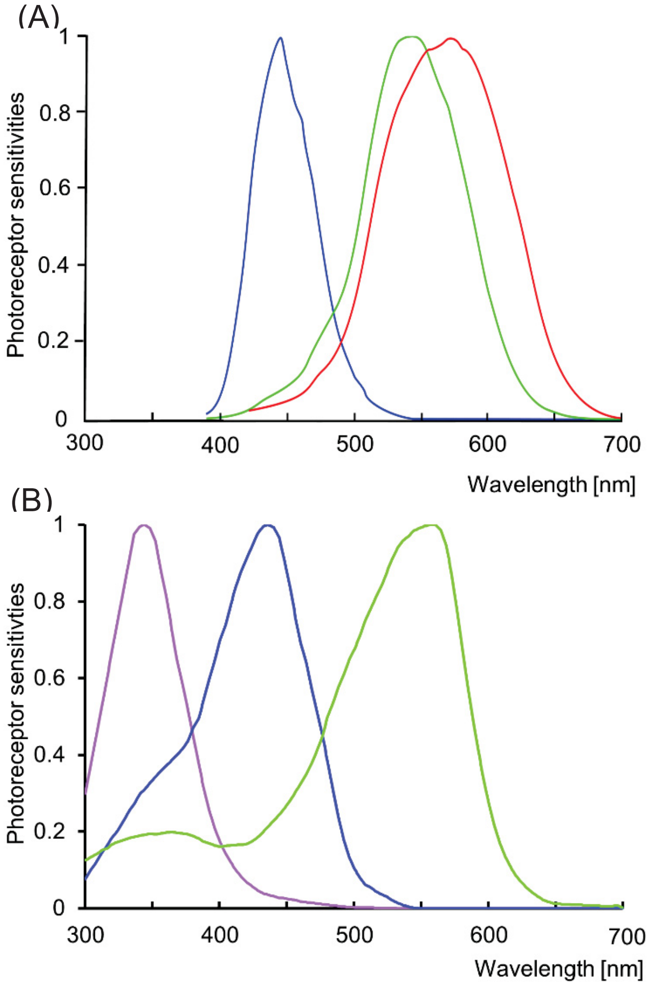

Fig. 2 Photoreceptor sensitivities of humans (A) and the Western honey bee (B).

colour vision system of bees (Menzel et al. 1986; Backhaus 1991; Chittka 1992; Vorobyev & Osorio 1998). Like humans, bees have a trichromatic visual system (Fig. 2B), with the difference that unlike humans, bees lack a red-sensitive photoreceptor type. In addition to the blue- and green-sensitive photoreceptor types, bees possess an ultraviolet-sensitive photoreceptor type. Especially the colour vision of the economically important Western honey bee (*Apis mellifera*) has been in the focus of researchers and advanced to be a model organism for colour vision research. Another thoroughly studied bee species is the Buff-tailed bumble bee (*Bombus terrestris*) that is easily managed in the lab and is used for greenhouse pollination of buzz-pollinated plants like tomato. The discovery of colour vision in bees by Karl von Frisch was only the stepping stone of research that analysed bees' abilities for the detection, recognition, cognition and preference of colours.

A specific characteristic of colour vision in bees is the angular dependence of chromatic vision. Bees use their green receptor to perceive achromatic information, also known as green contrast, while all three photoreceptor types are involved when chromatic vision is used (Giurfa et al. 1996, 1997). Especially while flying, achromatic information are used to compensate image motion during flight (Lehrer 1987, 1993). Achromatic vision is solely impacted by brightness contrasts, while the spectrum of colour composition is neglected (Giurfa et al.1999; Hempel der Ibarra et al. 2000). But in order to perceive these achromatic information the visual angle must be above 5° in honey bees. Between visual angles of 5° - 15° achromatic information is perceived and above 15° honey bees perceive chromatic information (Giurfa et al. 1996, 1997). The angles in which achromatic and chromatic information are received vary between different bee species. Buff-tailed bumble bees perceive achromatic information between 2.3° – 2.7° and chromatic information are perceived when the visual angle exceeds 2.7° (Dyer et al. 2008). Achromatic information is discussed to play an important role in long distance detection of floral targets, while chromatic information is more important in close range detection where chromatic patterns, e. g. floral guides, are important cues for bees (Lunau et al. 1996; 2006).

Colour has three main properties: hue, saturation and brightness. As previously mentioned, brightness plays an important role in terms of green contrast. Nonetheless, in bee vision brightness or intensity are assumed to play a minor role when it comes to colour perception (Chittka 1992; Vorobyev & Brandt 1997). Colour models, like the colour hexagon by Chittka (1992), do not take brightness into account when calculating bee-subjective colour traits. Studies conducted by Hempel de Ibarra (2000) and Telles and Rodríguez-Gironés (2015) suggest influences by brightness based on contrasts to the background, but these results are highly discussed (Ng et al. 2018).

That bees are influenced by colour hue has been proven in several studies. Honey bees and bumble bees both showed preferences for bluish colours, bumble bees additionally preferred yellow targets (Menzel 1967; Giurfa et al. 1995; Gumbert 2000; Raine & Chittka 2007; Dyer et al. 2008; Ings et al. 2009; Hudon & Plowright 2011; Morawetz et al. 2013; Avarguès-Weber & Giurfa 2014; Ostroverkhova et al. 2018).

The saturation of colours and its effect on colour choices of bees has moved into the focus of researchers (Lunau 1990; Rhode et al. 2013; Papiorek et al. 2013). Honey bees and bumble bees showed spontaneous preferences for colours with higher saturation than the colour they were trained to and independent of pigment concentration (Rohde et al. 2013; Papiorek et al. 2013).

The background against which a target is presented can influence the perception of the target. The resulting contrast between background and target colour are assumed to be important (Lotto & Chittka 2005). The way bees perceive colours can be changed depending on the colour and composition of the background (Bukovac et al. 2017). Bee-subjective calculation based on the colour hexagon by Chittka (1992) take the colour of the background into consideration.

An important factor that needs to be taken into consideration when working on colour choices of bees is their level of experience. Unexperienced flower workers that leave the hive for the first time are still able to find flowers in a relatively short amount of time, suggesting that they are driven by innate preferences which was first proposed by Darwin (1877). Many studies analysed the choice behaviour of naïve worker bees and found evidences for the existence of innate colour preferences (Menzel 1967; Lunau 1990, 1992; Lunau & Maier 1995; Giurfa et al. 1995; Lunau et al. 1996; Heuschen et al. 2005; Pohl et al. 2008; Morawetz et al. 2013). The preference of workers for bluish colours is an example for innate preferences exhibited by honey bees and bumble bees (Lunau et al. 1996; Raine et al. 2006a). The learning ability of bumble bees varies between hives showing the versatility created by learning experiences (Chittka et al. 2004; Raine et al. 2006b). Honey bees are known for their impressive cognitive capabilities and their ability to learn relations between different features of flowers, like colour, scent, and form (Pessotti 1981; Brown et al. 1998; Srinivasan et al. 1998; Zhang et al. 1999; Giurfa et al. 2001; Reinhard et al. 2004, 2006; Cooke et al. 2007; Moreno et al. 2012). Workers override innate or previously learned preferences with newly learned information, but the innate responses are not lost (Gumbert 2000). Based on learning experiences, bees prefer flowers according to nectar content rather than by colour (Pankiew 1967; Giurfa 1991; Greggers & Mauelshagen 1997).

The knowledge concerning colour vision in stingless bees is extremely sparse. Sánchez and Vandame (2012) tested the influence of colour and shape on the choice behaviour of *Scaptotrigona mexicana* Guérin and could show that colour has a stronger impact on their choices than shape. Furthermore, by utilising the method of Karl von Frisch (1914) the ability of colour vision and the ability of colour discrimination in stingless bees had been shown (Spaethe et al. 2014). But the results suggest that the colour discrimination of stingless bees is poorer than in honey bees and bumble bees.

Colour vision in the context of pollination

Flowering plants and animals, e. g. bees, butterflies, birds, and bats, established an ongoing relationship that shaped appearances and characteristics of both groups over time. Visual capabilities and floral colours are driving influences that build this relationship (reviewed by Van der Kooi et al. 2018). Studies concerning the spectral profiles of flowers in the Northern Hemisphere (Israel) and the Southern Hemisphere (Australia) suggest that the colours of many melittophilous flowers are closely connected to the visual system of bees (Chittka & Menzel 1992; Dyer et al. 2012; Shrestha et al. 2013). In particular, the reflectance of flowers changes at points of the spectrum where the sensitivities of two photoreceptors overlap and, thereby, improve the discrimination ability of the beholder for these colours (Chittka & Menzel 1992; Dyer et al. 2012).

It has been shown that many flowers with bluish colours offer a higher nectar reward than flowers of other colours (Giurfa et al. 1995; Raine & Chittka 2007). There are two possible explanations discussed, either the visual system, especially innate preferences for bluish floral colours of bees shaped the evolution of floral colours or the naturally occurring higher amount of nectar in these flowers shaped preferences of bees (Chittka & Wells 2004; Raine & Chittka 2007).

An example for the role of colour in plant-pollinator interactions are honest flower signals, where the colour of a flower changes depending on the availability of rewards. Floral colour change has many forms, like shifts of reflectance to other wavelengths (e. g. *Lythrum salicaria*) and adding or deducting signals (Ohashi et al. 2015). Analysis of reflectance spectra of colour changing flowers revealed that green contrast of unrewarding flowers remains high and, thereby, continues to add to the long-distance signal of the plant or inflorescence (Ohashi et al. 2015).

Floral colour impacts foraging strategies of bees. A strategy used by many bee species is flower constancy – workers constantly visit flowers of the same species over a period of time although other species offer equivalent amounts of reward (Ribbands 1953; Heinrich 1975; Waser 1983, 1986; Wells & Wells 1983; Real 1991). Wells and Wells (1983, 1984, 1986; Hill et al. 1997) offered blue and yellow coloured artificial flower dummies to honey bee workers that had no prior training, and were guided by olfactory cues. Individual workers either continuously visited yellow or blue flower dummies even when they differed in quality, quantity and frequency of reward (Hill et al. 1997). Workers can learn differences between colour morphs very easily, but when not trained to visit different colour morphs most workers chose one morph and visit it with great fidelity, without sampling other morphs (Free 1963; Moezel et al. 1987). Stingless bees behave similar to honey bees when tested for flower constancy (Slaa et al. 1998a, b, 2003). The only difference found in *Oxytrigona mellicolor* was that workers were influenced by the presence of nestmates, but probably this is related to species-specific foraging behaviour (Slaa et al. 2003).

Outlook of this dissertation

This dissertation bases on three articles, published in international peer-reviewed journals and two articles under review. The focus is on colour preferences of stingless bees, a so far understudied tribe of eusocial bees. The results are put into ecological context by comparing them to previously published results concerning colour preferences of other eusocial bees, including collected data of *Apis mellifera*, which is a model organism for colour vision in bees.

First, innate colour preferences of the Australian native stingless bee *Tetragonula carbonaria* are tested under laboratory conditions by using standard broadband reflectance stimuli that are representative for common flower colours (Dyer et al. 2016). Colour signals of flowers are assumed to be equally distributed in the colour space of bees and that the relationship between bees as pollinators and flowers as food providers formed the fit between the visual signals of flowers and the colour perception of bees. If that is the case, bees around the world should show similar responses and preferences for colours. Honey bees and bumble bees prefer bluish colour hues and saturated colours. In Chapter 2, the aim was to analyse the choice behaviour of *T. carbonaria* concerning the colour parameters hue, saturation and green contrast.

For the next three experiments (Chapter 3, 4 and 5), a newly developed method, in which pigment powders are mixed to manipulate single colour parameters is utilized, resulting in fine scaled differences of colour parameters. The aim of all three experiments is to analyse the impact of the colour parameters brightness, saturation and hue on the choice behaviour of stingless bees. Two Brazilian stingless bee species (Chapter 3: *Melipona mondury*, *Melipona quadrifasciata*) are tested regarding their colour preferences for all three colour parameters and the impact of differing background colours (Koethe et al. 2016). Individual workers choose stimuli with different hues or same hues but with altering brightness and saturation values. In Chapter 4, the same method is used, but the colour stimuli are calculated based on the colour hexagon by Chittka (1992) that refers to bee-subjective colours (Koethe et al. 2018). Again, two Brazilian stingless bee species (*Melipona bicolor* and *Partamona helleri*) are tested concerning their colour preferences. In the last experiment using the colour pigment method, *T. carbonaria* and the Western honey bee, *Apis mellifera*, have to choose between blue stimuli that either differ regarding their brightness or their saturation. The Chapters 2 - 5 comprise experiments that analyse colour preferences in stingless bees, with an emphasis on single colour parameters. In Chapter 6, an experiment is conducted to analyse the food source selection of two Brazilian stingless bee species (*Melipona subnitida* and *Plebeia flavocincta*) and the Western honey bee. The aim is to see what factors besides colour influence selection of food sources in eusocial bees. Factors that are analysed in this experiment are scent cues deposited by conspecifics, colour of food sources, distance between food source and hive, and learned location based on training.

The results of all five publications are discussed based on experience of foragers, environmental impacts on colour vision, behavioural aspects (e. g. recruitment, division of labour and foraging strategies) and morphological differences.

CHAPTER 2

This chapter has been published as:

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

Dyer AG, Boyd-Gerny S, Shrestha M, Lunau K, Garcia JE, Koethe S, Wong BBM

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

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The findings of this study were reported at the 11th International Congress of Neuroethology, Sapporo, Japan, 1st August 2014 as part of the symposium 'In the footsteps of Karl von Frisch: 100 years of investigation into colour and polarization vision'.

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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 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 Snap-dragons (*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 broad-band 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 ± 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 × 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 × 20 × 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.

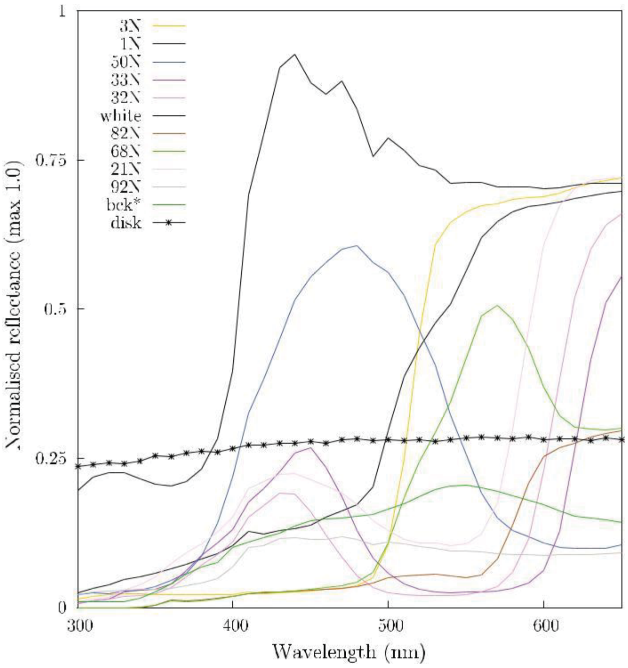

Fig. 1 Spectral reflectance of the 10 HKS colour stimuli, the *green background* (bck) and the aluminium disc priming stimuli. Normalised data plotted relative to a calibrated white $BaSO_4$ standard (Ocean Optics) that reflects radiation equally from 300 to 650 nm

Flight arena dimensions were 1.2 × 0.6 × 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, 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,

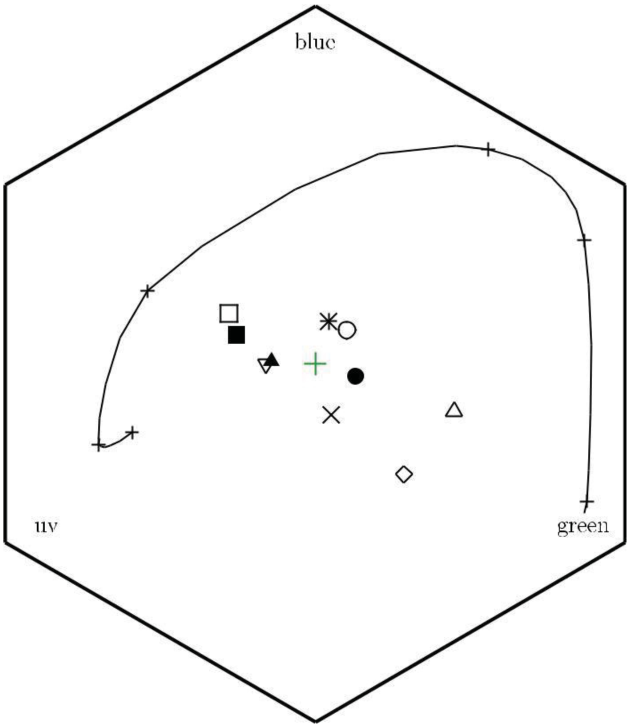

Fig. 2 Plots of HKS colour stimuli on hexagon space for *Tetragonula carbonaria* bee vision: 3N (*diamond*), 1N (*multiplication sign*), 50N (*asterisk*), 33N (*open square*), 32N (*filled square*), white (*open circle*), 82N (*filled circle*), 68N (*open upward triangle*), 21N (*filled upward triangle*) 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 tics on spectral locus indicate wavelengths from 350 to 550 nm at 50 nm intervals following methods in Chittka and Kevan (2005)

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 Kooi 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 alphaband 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 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 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.

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] Sensu (Spaethe et al. 2001)

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* 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).

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**	**0156**	**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 *α* = 0.05

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 (rs) = −0.669, *P* value = 0.034], and between spectral purity and chromatic contrast (rs = 0.766, *P* 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* value = 0.004; Ggreen contrast × purity = 8.95, *P* 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* value = 0.768). Individual plots for the main effects and the interaction term are depicted in Fig. 3.

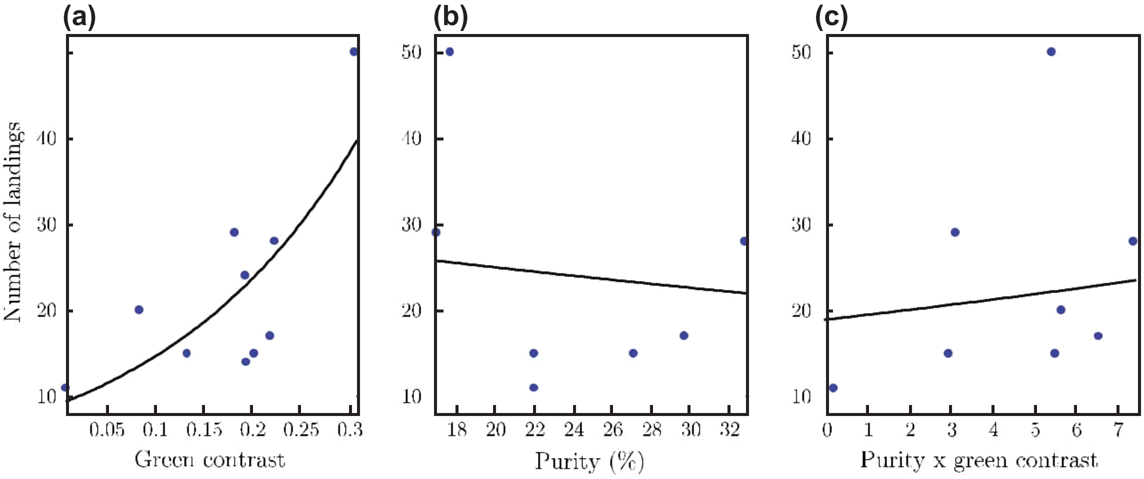

Fig. 3 Analyses of innate colour preference choices for colony 1 of the stingless bee *T. carbonaria*. Regression models (*solid lines*) for the main effects and interaction term of the regression model explaining the relationship between number of observed landings (*y* axis) and various colour characteristics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

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 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).

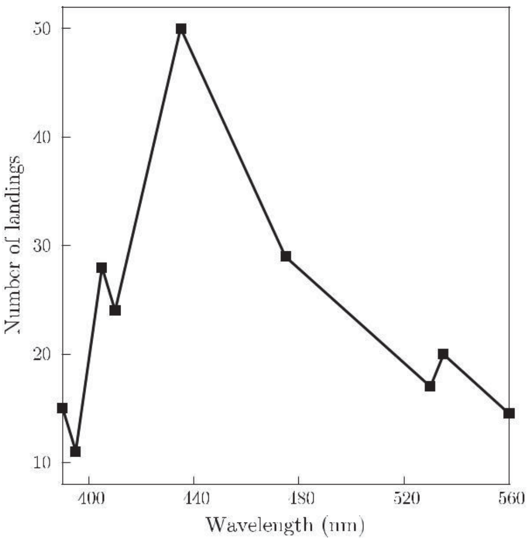

Fig. 4 Number of choices by stingless bee *T. carbonaria* (colony one) plotted against the dominant wavelength corresponding to each of the *different colour* targets used for the experiment.
Dominant wavelength values were obtained assuming chromatic adaptation to a *green background* as per Chittka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

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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CHAPTER 3

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Colour is more than hue: preferences for compiled colour traits in the stingless bees *Melipona mondury* and *M. quadrifasciata*

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Abstract

The colour vision of bees has been extensively analysed in honeybees and bumblebees, but few studies consider the visual perception of stingless bees (Meliponini). In a five stage experiment the preference for colour intensity and purity, and the preference for the dominant wavelength were tested by presenting four colour stimuli in each test to freely flying experienced workers of two stingless bee species, *Melipona mondury* and *Melipona quadrifasciata*. The results with bee-blue, bee-UV-blue and bee-green colours offered in four combinations of varying colour intensity and purity suggest a complex interaction between these colour traits for the determination of colour choice. Specifically, *M. mondury* preferred bee-UV-blue colours over bee-green, bee-blue and bee-blue-green col-ours while *M. quadrifasciata* preferred bee-green colour stimuli. Moreover in *M. mondury* the preferences were different if the background colour was changed from grey to green. There was a significant difference between species where *M. mondury* preferred UV-reflecting over UV-absorbing bee-blue-green colour stimuli, whereas *M. quadrifasciata* showed an opposite preference. The different colour preferences of the free flying bees in identical conditions may be caused by the bees' experience with natural flowers precedent to the choice tests, suggesting reward partitioning between species.

Keywords Stingless bees · *Melipona* · Colour intensity · Spectral purity · Dominant wavelength

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Introduction

Bees are important pollinators of flowering plants (Batra 1995) and valued in the pollination management of agricultural crops (Potts et al. 2010). Worldwide, around 70 % of crops cultivated for human consumption depend on pollination by insects, mostly bees (Klein et al. 2007). Social bees like *Apis mellifera* and *Bombus terrestris* are easily kept in captivity in great numbers and thus serve as important pollinators. Also stingless bees readily accept hives and thus can be used for pollination management. Choice of food plants in bees is not only influenced by decisions related to floral rewards and risks, i.e. nectar concentration and volume of flowers, predation risk and distance between hive and food source. It also depends on abilities of bees to reduce their search time (Spaethe et al. 2001; Rodriguez-Gironés and Santamaría 2004) and to memorize flowers, as well as on innate and learnt preferences to find rewarding flowers (Marden and Waddington 1981; Simonds and Plowright 2004; Cnaani et al. 2006; Reader et al. 2006).

Bees recognize flowers based on multimodal cues (Giurfa and Lehrer 2001; Horridge 2005; Raguso 2008). In addition to olfactory cues (Chittka and Raine 2006) as well as morphological cues like shape (Lehrer et al. 1995) and size

(Spaethe et al. 2001; Avarguès-Weber et al. 2014) of flowers, colour wields a major influence on the choice behaviour of bees (von Frisch 1967). The foraging strategies of bees including flower constancy result in selective pressure on plants to adapt flower signals to the pollinators' visual system (Chittka and Menzel 1992; Chittka 1996; Chittka and Raine 2006; Dyer et al. 2012).

Flower colours comprise numerous cues that must be taken into consideration to understand colour choices in bees: Besides colour hue (Giurfa et al. 1995; Horridge 2007), colour saturation (Lunau 1990; Papiorek et al. 2013; Rhode et al. 2013), chromatic contrast against background (Giurfa et al. 1995; Lunau et al. 1996; Spaethe et al. 2001) and green contrast (Giurfa et al. 1996, 1997; Dyer et al. 2008) influence the colour choice behaviour in bees. Brightness is typically considered to be less important for the bees' choice and perception of colours (Srinivasan and Lehrer 1984; Backhaus 1991; Vorobyev and Brandt 1997; Hempel de Ibarra et al. 2000; Spaethe et al. 2001), although this factor remains to be thoroughly tested.

In addition to contrast between colours (Lehrer and Bischof 1995; Lunau et al. 1996; Dyer and Chittka 2004; Dyer et al. 2008), three main colour traits of coloured stimuli have been established in experiments of colour preferences in bees: dominant wavelength (referring to colour hue), spectral purity (referring to colour saturation) and colour intensity (referring to brightness) (Daumer 1956; Menzel 1967; Lunau 1990; Lunau and Maier 1995; Lunau et al. 1996; Hill et al. 1997; Gumbert 2000). Colour choice experiments in bees can reveal deviant results in dependence of the experimental design, and so far only few studies have considered the complexity of how colour traits may interact to influence bees' decision making (Spaethe et al. 2001; Morawetz et al. 2013).

Innate colour preferences are studied in tests with flower-naïve and non-trained bees (Lunau et al. 1996). Spontaneous colour preferences are found in tests with bees subjected to minor pretraining and may demonstrate preferences for colours, which differ from those of trained bees (Lynn et al. 2005; Rhode et al. 2013; Papiorek et al. 2013). In colour discrimination tests with bees, learned colour preferences can overwrite innate preferences and depend on absolute or differential conditioning (Gumbert 2000; Giurfa 2004; Morawetz et al. 2013). None of these studies could unveil which colour trait has the strongest impact on colour choice in bees. In general, some studies found preferences for colour stimuli with a dominant wavelength in the blue wavelength band in bees (Menzel 1967; Giurfa et al. 1995; Gumbert 2000; Raine and Chittka 2007; Dyer et al. 2007; Ings et al. 2009; Hudon and Plow-right 2011; Morawetz et al. 2013). A less distinct preference seems to exist for colour stimuli with a dominant wavelength in the yellow wavelength band (Giurfa et al. 1995; Gumbert 2000). Besides the dominant wavelength of colours the spectral purity of colour stimuli was identified to have a strong impact on colour choice in bees (Lunau 1990; Lunau et al. 1996; Papiorek et al. 2013). The impact of colour intensity is controversial. Most studies assume no impact caused by colour intensity (Chittka 1992; Vorobyev and Brandt 1997), although experimental analyses are rare (Daumer 1956; Backhaus 1991), while Hempel de Ibarra et al. (2000) found, based on experimental data, that the intensity contrast between floral colour and background has an influence on the choice by bees. In addition Telles and Rodríguez-Gironés (2015) found an effect of brightness difference on the colour discrimination ability of bumblebees. It just seems justified to study the contribution of the intensity of colour stimuli for colour preferences in bees in general and in stingless bees.

Up to now the study of bee colour vision and colour preferences according to distinct colour traits has been focused on only two species, the Western Honeybee, *Apis mellifera*, and the Bufftailed Bumblebee, *Bombus terrestris*. The general attributes of colour vision, e.g. the spectral sensitivity of the photoreceptor types, are available for a large number of bee species, including four species of stingless bees (Peitsch et al. 1992). These findings indicate that bees use similar prerequisites such as a trivariant colour vision system (Chittka 1996; Briscoe and Chittka 2001). Despite the outstanding role of stingless bees as pollinators in the Paleotropics, Neotropics and Australis as well as their large ecological diversity, the colour vision and colour preferences of stingless bees are severely understudied (Slaa et al. 2006). In fact there is only a single and preliminary study about colour preferences in stingless bees (Sánchez and Vandame 2012). Quite recently, Spaethe et al. (2014) proved colour vision in the stingless bees *Trigona* cf. *fuscipennis* and *Tetragonula carbonaria*. But for many regions around the world we have a poor understanding at present about how these potentially important pollinators sense flowers.

The aim of this study is to compare the influence of the three main colour traits on the colour choice in two stingless bee species of the tribe Meliponini. We developed a new experimental design to study the colour choice behaviour of freely flying workers of stingless bees. We present a significant advancement for testing bee broadband colour perception by manufacturing of colour stimuli using different amounts of coloured, white, grey, and black pigment powders to systematically vary the colour traits dominant wavelength, colour purity and colour intensity. The experimental design facilitates each individual bee to experience five quadruple choice tests. The rationale of this experimental procedure is to test for hypothetical preferences of combinations of slightly more or less intense and slightly more or less pure colours (tests I–IV). Finally we test for hypothetical preferences for one of four colour stimuli each with a distinct dominant wavelength corresponding to a bee-subjective colour hue (bee-blue-green, bee-blue, bee-UV-blue, and bee-green), and thus representing the most common bee-colours of melittophilous flowers (Chittka and Menzel 1992). For this initial study of colour preferences in stingless bees the colour stimuli were manufactured based on the differences in the spectral reflectance properties of the mixed pigment powders, whereas assumptions about the spectral sensitivities of the photoreceptors and modelling of colour vision were excluded at that time.

The colour preferences of workers of *M. mondury* and *M. quadrifasciata* were tested to address the following questions: How do the tested colour traits, dominant wavelength, spectral purity and colour intensity, influence the choice of foraging bees? Do the bees exhibit a preference for one of the four tested dominant wavelengths? Does the background against which the artificial flowers are presented influence the choice? Is a single colour trait or a combination of certain traits determining the bees' colour choice behaviour? Are there differences in colour choice between the two *Melipona* species?

Materials and methods

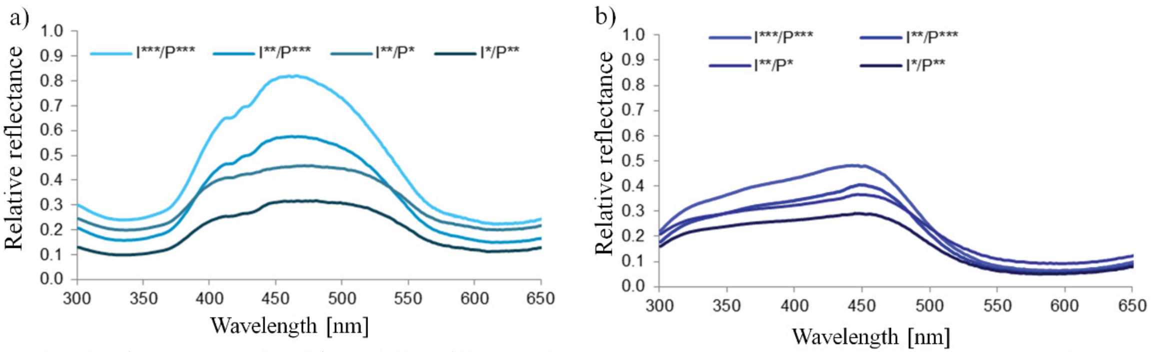
Bee keeping

The experiment took place on the campus of the UFPR (Universidade Federal Do Paraná) in Curitiba, Brazil, where two species of stingless bees *M. mondury* and *M. quadrifasciata* (Meliponini, Apidae) were tested. We used two colonies of each species which were flying freely and thereby gained experience with food plants.

Artificial flowers

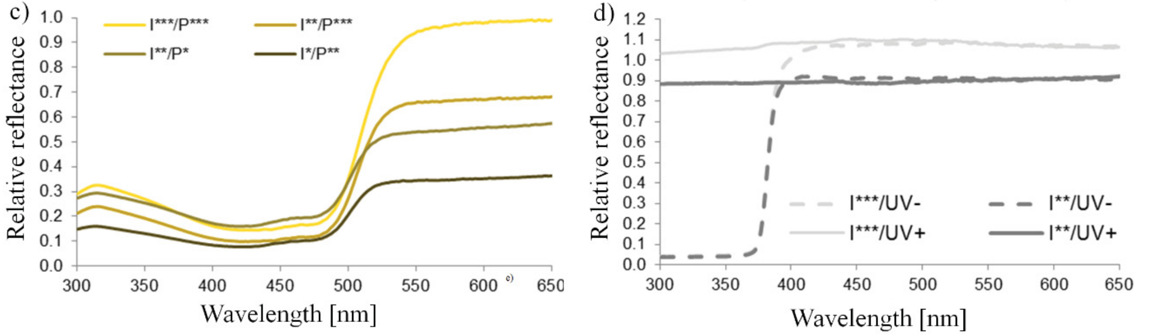
A new method to manipulate specific colour traits of artificial flowers was developed, which is based on the mixture of pigment powders. So called ‘basic pigments’ were used to determine the dominant wavelength (Artist Pigments: “Sky Blue”, “Ultramarine Blue”, “Yellow” and “Zinc White”, Art Material International Warenhandelsgesellschaft mbH, Kaltenkirchen, Germany; powdered sugar, Diamant, Pfeifer and Langen GmbH & Co. KG, Cologne, Germany). Two white powders—the pigment “Zinc White” and powdered sugar—were necessary to produce a UV-reflecting (“Zinc White”) and a UV-absorbing (powdered sugar) colour. For the manipulation of colour intensity and colour purity, barium sulphate (white), black pigment or a mixture of both powders were added (barium sulphate 99 % pure, Grüssing GmbH Analytika, Filsum, Germany; Artist Pigment “Black 722”, Art Material International Warenhandelsgesellschaft mbH, Kaltenkirchen, Germany). The powders were pressed into a culture dish (35 mm in diameter, 10 mm high) with a spatula. Various combinations of colour intensity and colour purity were fabricated by mixing defined amounts of the basic powder and varying amounts of white, grey and/or black powders. The resulting colours were measured via spectrometer analysis (USB4000 miniature fibre optic spectrometer, Ocean Optics GmbH,Ostfildern, Germany) at an angle of 45° using a UV-NIR deuterium halogen lamp (DH 2000-BAL, Ocean Optics GmbH), which was connected to the spectrometer by a UV–VIS fibre optic cable (Ø 600 µm, QR600-7-UV 125 BX, Ocean Optics GmbH). Barium sulphate was used as white standard while a piece of black cardboard served as black standard.

To determine the pigment mixtures of each stimulus used in the experiments the dominant wavelength, colour purity and colour intensity were calculated according to Valido et al. (2011); these values, however, were only used to check the differences in the spectral reflectance properties of the test stimuli. The simple formulas of Valido et al. (2011) were used to categorize the colour stimuli according to intensity and colour purity, thereby using colour variables that lack any bias in regard to a distinct spectral sensitivity of photoreceptors, spectral distribution of ambient light during the tests, and tested bee species. Several colour vision models have been proposed to understand bee colour vision, but there is no general agreement about which one should be given preference (Hempel de Ibarra et al.



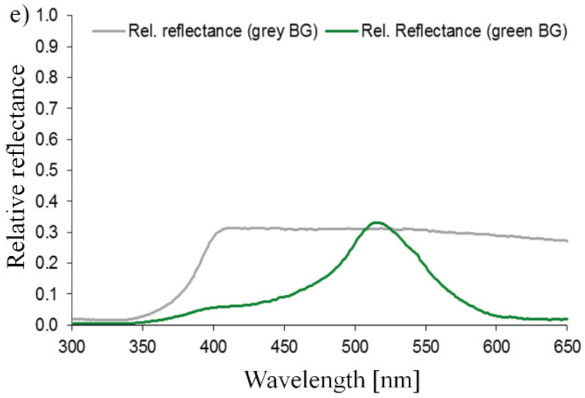
Stage I	Dominant wavelength	Intensity/1000	Colour purity
I***/P***	462	17.20	1.45
I**/P***	480	12.16	1.46
I**/P*	477	12.12	0.89
I*/P**	466	7.54	1.20

Stage II	Dominant wavelength	Intensity/1000	Colour purity
I***/P***	443	10.00	1.73
I**/P***	448	8.33	1.74
I**/P*	447	8.84	1.29
I*/P**	445	6.71	1.49



Stage III	Dominant wavelength	Intensity/1000	Colour purity
I***/P***	700	22.71	1.55
I**/P***	700	15.98	1.55
I**/P*	700	15.36	1.18
I*/P**	700	9.25	1.35

Stage IV	Dominant wavelength	Intensity/1000	Colour purity
I***/P***	525	34.36	1.27
I**/P***	418	29.34	1.25
I**/P*	445	42.93	0.07
I*/P**	695	36.03	0.05



	Dominant wavelength	Intensity/1000	Colour purity
Grey BG	445	9.55	1.30
Green BG	515	3.39	4.00

Fig. 1 Spectral reflectance curves and calculated colour traits of the colour stimuli and the background colours. The percentage of reflectance of the test stimuli of the four stages is plotted against the wavelength from 300 to 700 nm. **a** Bee-blue test stimuli with a dominant wavelength in the blue range of wavelength (stage I). **b** Bee-ultraviolet-blue test stimuli with a dominant wavelength in the blue range of wavelength, but additional reflectance in the ultraviolet range of wavelength (stage II). **c** Bee-green test stimuli with a dominant wavelength in the yellow range of wavelength (stage III). **d** Bee-blue-green test stimuli, either UV-reflecting or UV-

absorbing (stage IV). **e** Spectral reflectance of the two background colours. Calculated colour traits (dominant wavelength, colour intensity and colour purity): *I* = colour intensity; *P* = colour purity; *UV*− = ultraviolet-absorbing; *UV*+ = ultraviolet-reflecting; * = low; ** = medium; *** = high

2014; Kemp et al. 2016). For the evaluation of colour parameters as perceived by the stingless bees the bee-subjective colour parameters were calculated using the colour hexagon (Chittka 1992) based on the spectral photoreceptor sensitivities of *M. quadrifasciata* (Peitsch et al. 1992).

The dominant wavelength is defined as the maximum reflectance value [$\lambda(R_{\max})$]. Colour intensity is defined as the cumulative sum of all reflectance values between 300 and 700 nm [$\Sigma R(\lambda 300–700)$], and colour purity is defined as the difference between the minimum and maximum reflectance values divided by the mean reflectance value [$(R_{\max}–R_{\min})/R_{\mathrm{mean}}$] (Valido et al. 2011). The calculated colour traits of a great number of mixtures were compared and four mixtures per dominant wavelength were chosen for the experiments according to similar differences in colour purity and colour intensity (Fig. 1). The selected stimuli were labelled *I**/*P*** (low intensity, medium purity), *I***/*P** (medium intensity, low purity), *I***/*P**** (medium intensity, high purity), and *I****/*P**** (high intensity, high purity).

Experimental setup

The first step of experimental testing was to habituate the stingless bees to the test arena made of cardboard (90 × 90 × 40 cm) coated with grey PVC foil (074 “mittelgrau”; ORACAL® 631 Exhibition Cal; Orafol; Oranienburg, Germany) or, alternatively, with green PVC foil (061 “grün”; ORACAL® 631 Exhibition Cal; Orafol; Oranienburg, Germany). During pretraining the bees were fed a sucrose solution (40–80 %, depending on the bees’ motivation) placed in the centre of the arena (Fig. 2a). The feeder offering the sucrose solution was coated with PVC foil of the same colour as the arena. As *M. quadrifasciata* needed an additional olfactory stimulus for orientation, vanillin sugar (1 % vanillin, 99 % sugar, added to the sucrose solution) was used to initially guide the bees to the feeder. After a voluntary return to the feeder each bee was individually labelled with Tipp-Ex® Ecolutions (Société Bic, Clichy, France). During pretraining the bees were fed a sucrose solution (40–80 %, depending on the bees' motivation) placed in the centre of the arena (Fig. 2a). The feeder offering the sucrose solution was coated with PVC foil of the same colour as the arena.

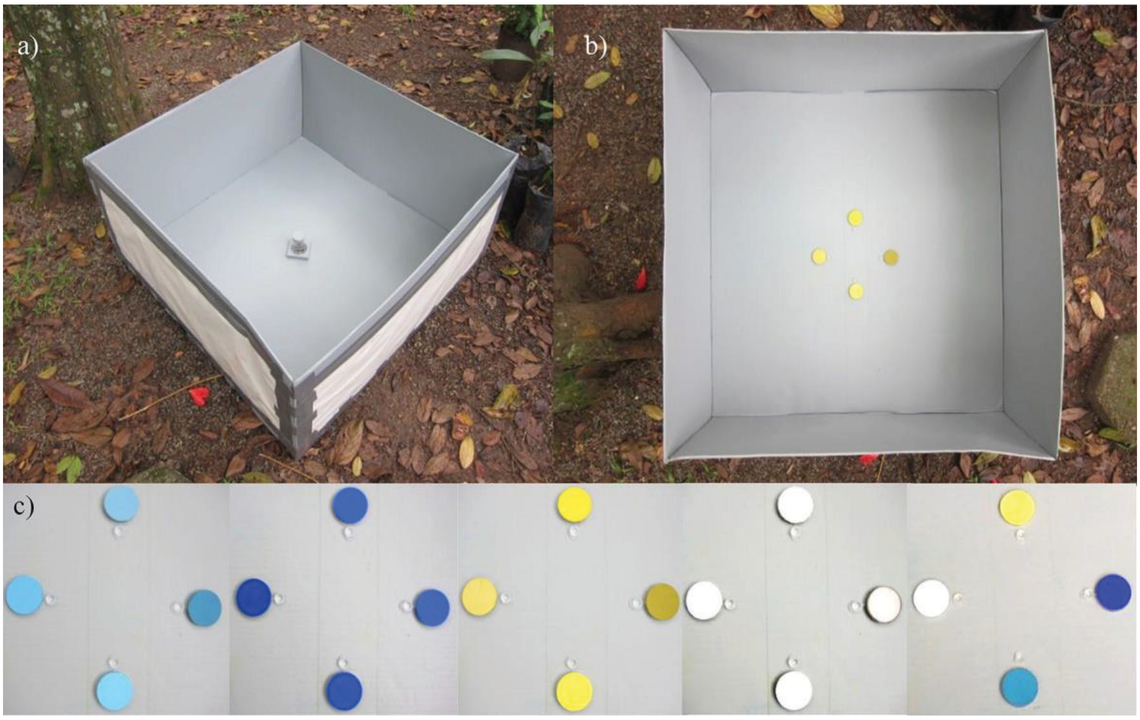


Fig. 2 Experimental setup and tested colour stimuli of the five stages. **a** The grey arena with the feeder as used during the pretraining phase. **b** The grey arena with the four colour stimuli and feeders were presented to the bee at the same time. Stage V was added to analyse the preferred dominant wavelength of the bees. Thus, all tests offered a quadruple choice to the tested bee as used during the colour choice experiments. **c** Overview of the five colour stages (I–V from *left* to *right*) used in the experiments. The same experimental stages were used for tests with the green arena.

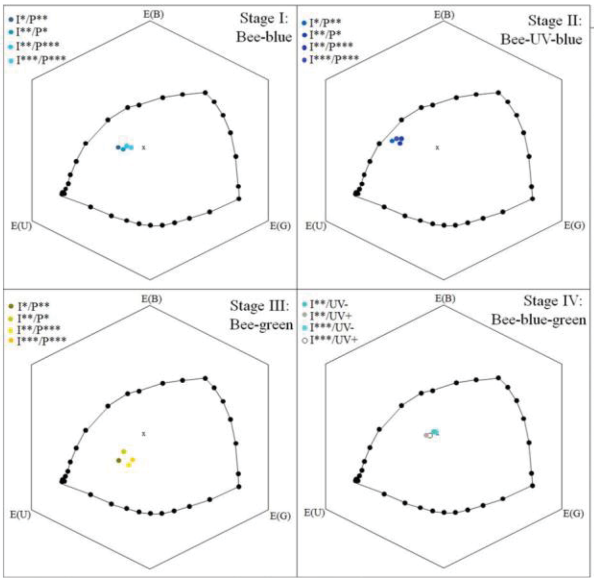
As *M. quadrifasciata* needed an additional olfactory stimulus for orientation, vanillin sugar (1 % vanillin, 99 % sugar, added to the sucrose solution) was used to initially guide the bees to the feeder. After a voluntary return to the feeder each bee was individually labelled with Tipp-Ex® Ecolutions (Société Bic, Clichy, France).

In the next step the feeder was replaced with four colour stimuli of one of the five stages (Fig. 2c) in the centre of the arena with 10 cm distance to the neighbouring stimuli (Fig. 2b). The transparent lid of a CapLock™ 1.5 ml tube (VWR International GmbH, Darmstadt, Germany) containing sucrose solution (with vanillin sugar for *M. quadrifasciata*) for the bees was placed next to each stimulus.

Each bee was tested in five stages and had to achieve 16 visits for each stage. The cap contained enough sucrose solution for the bees to forage *ad libitum* and return to the colony after each visit. To prevent position preferences the position of the stimuli was changed after every four visits using a pseudo random code, and the sequence of the first four stages was also randomised. Due to the fact that stingless bees use olfactory stimuli as orientation aids and bees are known to leave olfactory footprints (Jarau et al. 2004; Barth et al. 2008), the arena and the culture dishes were cleaned with a mixture of water and detergent after each test and the upper layer of the pigment was renewed. When a bee had finished stage I to IV a fifth stage V was arranged, in which the preferred stimuli of the stages I, II, III and IV were presented to the bee at the same time. Stage V was added to analyse the preferred dominant wavelength of the bees. Thus, all tests offered a quadruple choice to the tested bee.

Data analysis

The bee-subjective colour parameters, i.e. receptor specific contrast, colour contrast, green contrast, and spectral purity were calculated using the colour hexagon (Chittka 1992; Fig. 3, Online Resource 1/Table 1). In stages I–IV the aforementioned combinations of colour stimuli were tested for the four different dominant wavelengths, respectively bee-subjective colour hues: stage I bee-blue (basic pigment “Sky Blue” with a dominant wavelength around 470 nm), stage II bee-UV-blue (basic pigment “Ultramarine Blue” with a dominant wavelength around 445 nm), stage III bee-green (basic pigment “Yellow” with a dominant wavelength around 700 nm), and stage IV bee-blue-green (basic pigments “Zinc White” and powdered sugar). Bee-blue-green stimuli are named after their ultraviolet reflectance properties. The UV-reflective stimuli might be termed bee-white due to the strong reflectance in the ultraviolet range of wavelengths. The data was collected for 20 individuals of *M. mondury* and 15 individuals of *M. quadrifasciata*. The pooled data was analysed by testing the bees’ choices for the different stimuli of each stage using a generalised linear mixed model (GLMM) (Bates et al. 2011). We used the “lme4” package of R (R Development Core Team 2008) to analyse the individual choices of the bees, which were assessed using GLMM with Poisson distribution of data and the best linear fit depending on akaike information criterion (AIC) score. For overall test we analysed the number of choices for each stimuli as fixed effect and each individual bee was given a number, and this numbering was used as random effect of the model.



Stimulus	Receptor specific contrast			Chromatic contrast	Spectral purity	Green contrast
	S	M	L			
Bee-blue I***/P***	0.93	0.89	0.77	0.15	0.30	0.27
Bee-blue I**/P***	0.92	0.83	0.68	0.19	0.37	0.18
Bee-blue I**/P*	0.91	0.85	0.70	0.20	0.38	0.20
Bee-blue I*/P**	0.86	0.76	0.59	0.24	0.46	0.09
Bee-UV-blue I***/P***	0.94	0.84	0.57	0.34	0.66	0.07
Bee-UV-blue I**/P***	0.93	0.80	0.55	0.36	0.70	0.05
Bee-UV-blue I**/P*	0.93	0.81	0.53	0.34	0.65	0.03
Bee-UV-blue I*/P**	0.91	0.76	0.47	0.39	0.76	-0.03
Bee-green I***/P***	0.91	0.67	0.77	0.21	0.35	0.27
Bee-green I**/P***	0.91	0.69	0.69	0.26	0.42	0.19
Bee-green I**/P*	0.88	0.59	0.70	0.22	0.31	0.20
Bee-green I*/P**	0.84	0.53	0.58	0.28	0.43	0.08
Bee-blue-green I***/UV-	0.92	0.92	0.86	0.06	0.13	0.36
Bee-blue-green I**/UV-	0.98	0.93	0.86	0.08	0.16	0.36
Bee-blue-green I***/UV+	0.92	0.91	0.84	0.10	0.18	0.34
Bee-blue-green I***/UV+	0.97	0.91	0.84	0.12	0.22	0.34

Fig. 3 Colour loci of the test stimuli of stage I–IV with grey background in the colour hexagon (Chittka 1992) with calculated values of the receptor-specific contrast, chromatic contrast, spectral purity and green contrast

Table 1 Mixture ratio of tested pigments (white = $BaSO_4$, black = ’Artist Pigment Black 722’, grey = blend of $BaSO_4$ (94.337 g) and ’Artist Pigment Black 722’ (5.663 g)

	Sky blue I***/P***	Sky blue I**/P***	Sky blue I**/P***	Sky blue I**/P**	Ultramarine blue I***/P***	Ultramarine blue I**/P***	Ultramarine blue I**/P*	Ultramarine blue I*/P**
Basic pigment	57.140 g	74.055 g	53.317 g	95.196 g	74.998 g	85.658 g	50.004 g	85.704 g
White	42.860 g	18.531 g	26.673 g	–	–	–	–	–
Black	–	–	–	4.804 g	–	8.598 g	–	14.296 g
Grey	–	7.414 g	20.010 g	–	25.004 g	5.744 g	49.996 g	–

	Yellow I***/P***	Yellow I**/P***	Yellow I**/P*	Yellow I*/P**	Zinc white I***/UV−	Zinc white I**/UV−	Powdered sugar I***/UV+	Powdered sugar I**/UV+
Basic pigment	61.530 g	98.999 g	44.442 g	93.003 g	99.933 g	99.207 g	100.000 g	99.967 g
White	38.470 g	–	33.331 g	–	–	–	–	–
Black	–	1.001 g	–	6.997 g	0.067 g	0.793 g	–	0.033 g
Grey	–	–	22.227 g	–	–	–	–	–

To test the distribution of choices between the four stimuli of each stage a multiple comparison of means has been done with the Tukey all-pair comparisons. For further analysis of the bees' choice behaviour the chromatic contrast between the colour loci and the background, the spectral purity as the distance between the colour loci to the background divided by the distance between the background and the spectral line and the green contrast (excitation of green receptor minus 0.5) were calculated (Fig. 3, Online Resource 1) according to the colour hexagon (Chittka 1992), considering the spectral sensitivity of *M. quadrifasciata* (Peitsch et al. 1992).

Results

M. mondury and *M. quadrifasciata* workers were tested 16 times for each of the five different stages to evaluate their preferences for distinct colour traits; thus each individual bee has been tested 80 times. In total 2800 choices were evaluated.

Colour choices in *M. mondury* were tested in the grey as well as in the green arena, while colour choices in *M. quadrifasciata* were tested only in the grey arena. *M. mondury* (*n* = 20) exhibited only few distinct preferences for certain colour traits when the stimuli were presented against the grey background (Fig. 4). *M. mondury* workers showed a significant preference for the colour stimulus *I**/*P*** displaying the highest spectral purity according to the colour hexagon in stage I (bee-blue) when tested on a grey background, while they exhibited no preference when tested against a green background (Online Resource 2) with which the stimuli exhibited lower and more similar spectral purity according to the colour hexagon. In stage II (bee-UV-blue) no clear preference for any of the stimuli were observed independently of the background colour. The bee-green stimuli (stage III) were not discriminated by the bees independently of the background colour. In the bee-blue-green stage (IV) which consisted of two levels of intensity which differed in their UV-reflecting properties, i.e. UV-absorbing vs. UV-reflecting, *M. mondury* showed significant preferences for the two UV-reflecting stimuli. This preference was only observed when the stimuli were presented against the grey background whereas in the tests with the green background the bees showed no preference in stage IV (Online Resource 4); with both backgrounds the UV-reflecting stimuli displayed a higher spectral purity according to the colour hexagon. The last stage (V), comprising the individually preferred colour stimuli of the four initial stages, *M. mondury* showed a strong preference for the bee-UV-blue colour while presented against both background colours. The preferred colour stimuli in stage I–IV were not outstanding for their green contrast according to the colour hexagon.

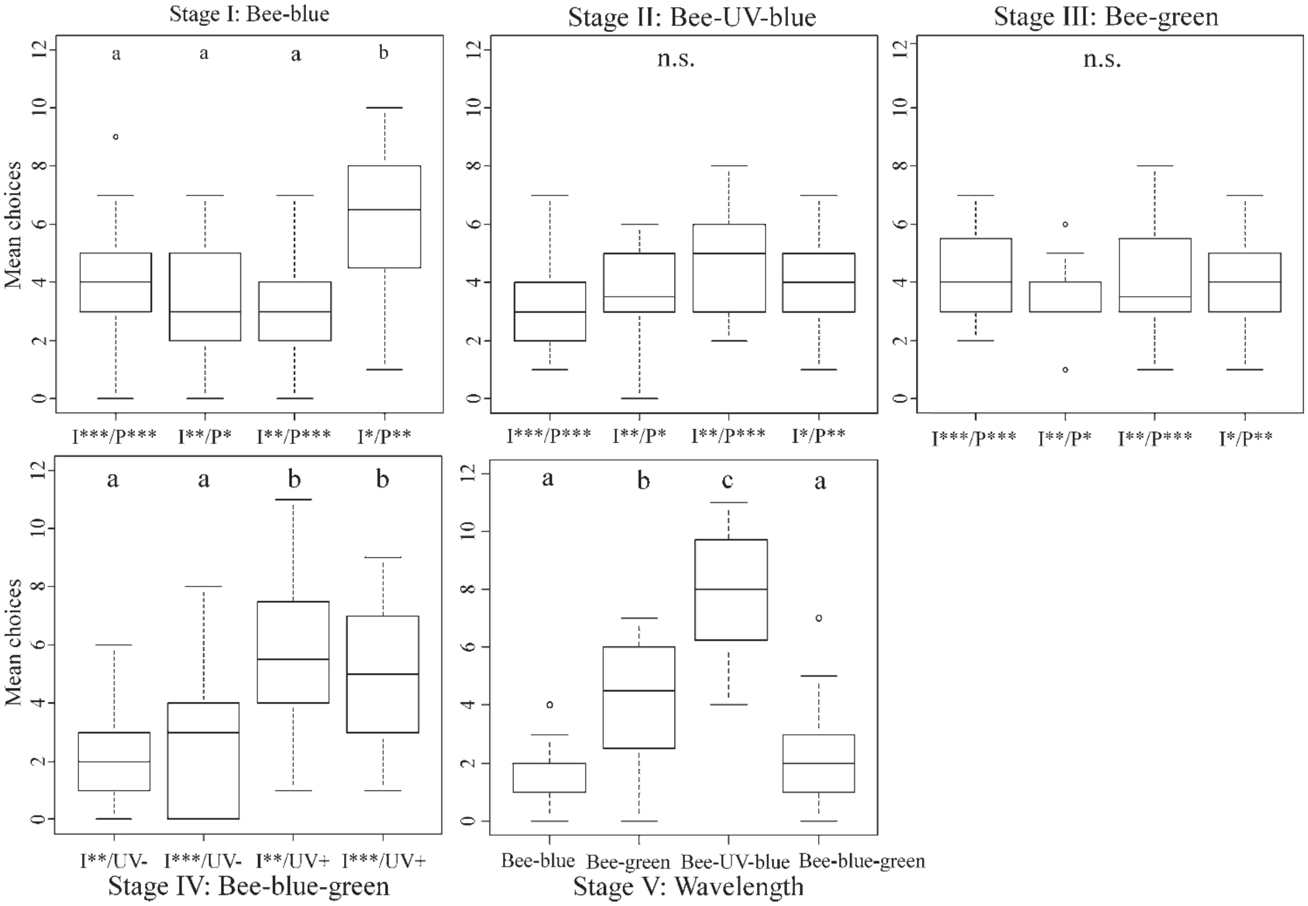


Fig. 4 Colour preferences of *Melipona mondury* tested with colour stimuli presented against *grey* background. The colour choices of the tested bees ($n = 20$) are plotted against the tested stimuli for every test stage. Workers were tested for bee-blue (stage I), bee-UV-blue (stage II), bee-green (stage III), and bee-blue-green (stage IV) colours. Subsequently the workers were tested for the most preferred colour hue (stage V). Every stage comprised four colours of the same dominant wavelength which varied in colour intensity and colour purity to test the impact of these two colour traits on the bees' colour choice. *I* = colour intensity, *P* = colour purity; * = low, ** = medium, *** = high; *UV−* = ultraviolet-absorbing; *UV+* = ultraviolet-reflecting. *Different letters* indicate significant differences, *n.s.* = not significant (Tukey all-pair comparisons)

M. quadrifasciata ($n = 15$) exhibited a different colour choice behaviour compared to *M. mondury* workers and restricted to tests with the grey background (Fig. 5). Bees did not choose differently between the stimuli of stage I (bee-blue), whereas their choice behaviour in stage II (bee-UV-blue) showed a slight preference of the stimuli with high spectral purity. In the bee-green stage (III) the *M. quadrifasciata* bees favoured none of the stimuli. A significant difference in the colour preference between the two *Melipona* species was found for the bee-blue-green stimuli (stage IV). *M. quadrifasciata* generally preferred UV-absorbing stimuli, particularly *I****/UV-, while *M. mondury* preferred the UV-reflecting bee-blue-green stimuli. In contrast to *M. mondury*, in stage V *M. quadrifasciata* bees exhibited a significant preference for the bee-green colour stimulus.

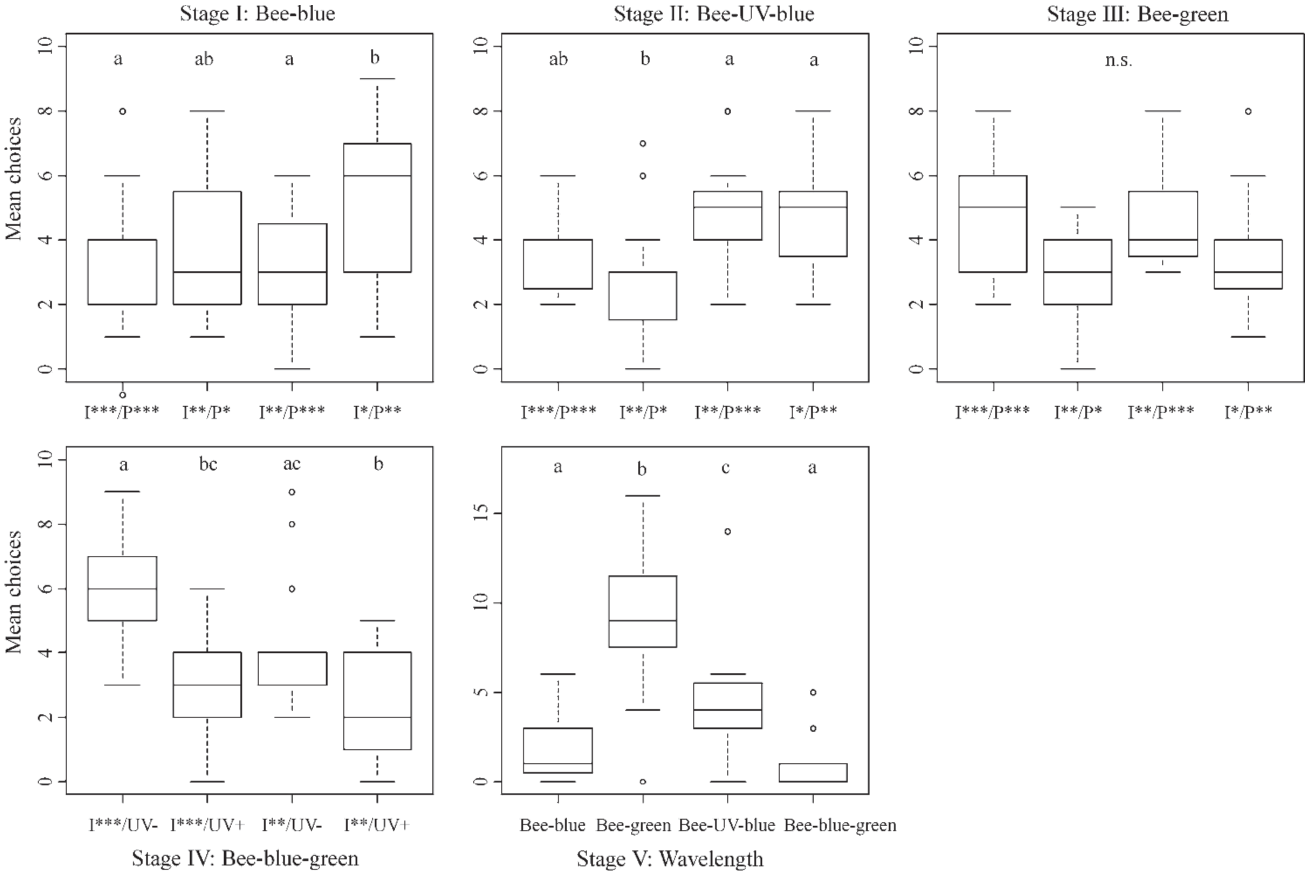


Fig. 5 Colour preferences of *Melipona quadrifasciata* tested with colour stimuli presented against a grey background. The colour choices of the tested bees (*n* = 15) are plotted against the tested stimuli for every test stage. Workers were tested for bee-blue (stage I), bee-UV-blue (stage II), bee-green (stage III) and bee-blue-green (stage IV) colours. Each stage comprised four colours of the same dominant wavelength which varied in colour intensity and colour purity to test the impact of these two colour traits on the bees' colour choice. I = colour intensity, P = colour purity; * = low, ** = medium, *** = high; UV- = ultraviolet-absorbing; UV + = ultraviolet-reflecting. *Different letters* indicate significant differences, *n.s.* = not significant (Tukey all-pair comparisons)

When only the most preferred test colour of each individual bee was evaluated, the resulting individual colour preferences are more clearly depicted. Individual *M. mondury* and *M. quadrifasciata* bees showed similar preferences for *I*/P*** bee-blue, *I***/*P**** bee-UV-blue, and *I****/*P**** bee-green colours presented against grey back-ground (Online Resource 3) indicating no preference for spectral purity or green contrast according to the colour hexagon. Individuals of *M. quadrifasciata* preferred UV-absorbing bee-blue-green colours over UV-reflecting ones, whereas *M. mondury* showed an opposite preference. *M. quadrifasciata* bees exhibited a clear preference for the dominant wavelength corresponding to the bee-subjective colour hue bee-green over the dominant wavelength corresponding to bee-UV-blue, whereas *M. mondury* exhibited an opposite preference; the individuals of both species largely ignored the colour stimuli displaying dominant wavelengths which corresponds to the bee-subjective col-our hues bee-blue and bee-blue-green (Online Resource 3).

Discussion

The results of the colour choice experiments unveiled differences in the colour choice behaviour between *M. mondury* and *M. quadrifasciata* workers. Neither of the two species showed a clear preference concerning spectral purity or green contrast; but *M. mondury* exhibited a preference for colours as defined by dominant wavelength or bee-subjective colour hue that was different to that of *M. quadrifasciata*. The experimental stages tested the bees' preferences for colours of different dominant wavelength in separate tests. The simultaneous offer of colour stimuli that display small differences in single colour traits has been successfully established by Rhode et al. (2013) using bumblebees and honeybees to demonstrate spontaneous preferences for colours of higher spectral purity. Although bees are able to discriminate between colours with chromatic contrast differences of about 0.05 hexagon units (Dyer et al. 2008; Spaethe et al. 2014), neither *M. quadrifasciata* nor *M. mondury* workers had a consistent preference for colour stimuli displaying a high spectral purity. It is unlikely that the different colour preferences in the two tested stingless bees are caused by deviant spectral sensitivity of the photoreceptor types, since the photoreceptor sensitivities in hymenopterans are highly conserved (Briscoe and Chittka 2001). However, previous work on bumblebees suggests intercolony differences in learning performance even within species (Raine and Chittka 2007); thus differences between related stingless bee species are not surprising.

Colour traits mediating bees’ colour choice

The colour preference in *M. mondury* was dependent of the background colour against which the test stimuli were presented. This result was unexpected, because the colour loci of the four colour stimuli presented simultaneously in stages I-IV were rather independent of the background in regard to contrast amongst the four stimuli (Fig. 3, Online Resource 1). However, the *M. mondury* bees did not prefer consistently colour stimuli displaying the largest chromatic contrast against the background. Moreover, in the stage V test, the colour hue preference of *M. mondury* workers was not affected by the background. Previous studies demonstrated that the perceptual chromatic contrast between the coloured stimuli and the background might influence the colour choice (Giurfa et al. 1995; Lunau et al. 1996). The finding that the value of chromatic contrast between the coloured stimuli and the background in our study does not predict choice preferences indicate that more than one colour trait mediates bee colour choices. The green background had strong reflectance in the long wavelength green region of the bee visual spectrum and low reflectance in the ultraviolet range of wavelengths and thus might have been more colourful to the bees as compared to natural backgrounds of green leaves (Chittka 1994, 1996). Also the grey background had low reflectance in the ultraviolet range of wavelengths and thus was colourful for the bees. It can thus not be excluded that the properties of the background colour might have affected the choice behaviour in an unforeseen manner.

Impact of background colour

The evaluation with the colour hexagon model assumes that the photoreceptors adapt to the background colour such that the excitation of the three photoreceptor classes is consistently half-maximal (Backhaus 1991; Chittka 1992; Vorobyev and Brandt 1997), which fits available physiological evidence in invertebrate vision (Laughlin 1981, 1989). Currently, however, it is not known whether the adaptation to artificial background colours with low/high UV reflectance may influence the assumed modelling of adaptation as compared to the natural backgrounds that are commonly employed to understand bee choices for colour in an ecologically relevant scenario.

The tested colour stimuli were chosen, since they meet the requirements of previously conducted studies (Chittka and Menzel 1992; Giurfa et al. 1995). Chittka and Menzel (1992) suggested that there may be an evolutionary adaptation of flower colours and the insect pollinators' colour vision systems: later it was found that the flower colours for flowering plants adapted to a phylogenetically ancient and conserved colour vision system in bees (Chittka 1996; Briscoe and Chittka 2001). In particular, it was shown that the sharp steps in spectral reflectance curves were located at ~400 and 500 nm. These marker points fit the turning points in the spectral reflectance curves of the bee-UV-blue and bee-green stimuli used in the current study, providing important insights into how stingless bees may also influence flower colour evolution.

Impact of foraging experience

Since the tested *Melipona* bees were freely flying before they were tested in the experimental setup, they potentially acquired colour preferences due to their foraging experience at natural flowers that might still have had an impact on the choice behaviour in the tests. The hives of the two bee species were kept in close proximity to each other which caused an identical spectrum of potential food sources and enabled a comparison between the two species. Since stingless bees are recruiting nestmates very effectively towards food plants (Jarau et al. 2000, 2003; Nieh 2004), the competition between the two species could have resulted in a partitioning of the food sources. Since we did not know the flower colour of the preferred natural food plants of the two stingless bee species at the time of experimental testing, it remains an unresolved question whether the significantly different preferences for UV-absorbing versus UV-reflecting bee-blue-green colours as well as those for bee-UV-blue versus bee-green colours are linked to learnt preferences from foraging experience at natural flowers. The studies of Gumbert (2000) and Rhode et al. (2013) showed that it depends on the number of training trials and on the similarity between training and test colours whether the bumblebees chose colours due to innate preferences. The influence of previously visited natural flowers cannot be excluded when working with freely flying bees. In addition, influences on choice behaviour by previous test stages can also be not excluded. Fact is that the choice of the preferred colour hue in stage V seemingly is not biased by the visits to rewarded bee-blue-green stimuli in stage IV, since the bee-blue-green colours were chosen least.

Ultraviolet reflectance

The preference of *M. mondury* for UV-reflecting bee-blue-green colours is of particular interest, since melittophilous flowers that appear white to humans regularly absorb ultraviolet light (Daumer 1958; Chittka 1994; Spaethe et al. 2001). The preference of bumblebees for UV-absorbing bee-blue-green colours over UV-reflecting ones has been interpreted to their preference for colours of high spectral purity (Lunau 1990, 1992; Lunau et al. 1996). Moreover Lunau et al. (2011) found that Neotropical white flowers that reflect ultraviolet light are pollinated by hummingbirds, whereas white flowers that absorb ultraviolet light are pollinated by bees. The predominance of bird-pollinated white UV-reflecting flowers in the Neotropics has been interpreted as a mechanism for sensory exclusion of bees corresponding to their opposing colour preferences (Lunau et al. 2011) and has been demonstrated recently in a natural setting (Bergamo et al. 2015). Bird-pollinated plants might benefit from the exclusion of nectar robbing bees and stingless bees are known for robbing bird-pollinated flowers (Roubik 1982). One might thus consider that *M. mondury* bees have evolved the ability to detect bird-pollinated flowers that are attempting to be cryptic to the bees. Personal observations during the tests indicate that *M. modury* bees learned faster to forage in the experimental setting as compared to *M. quadrifasciata* bees. This difference in learning speed might explain the difference of preference for UV-absorbing and UV-reflecting colours to avoid shared food sources. It seems an interesting idea that nectar robbing at bird-pollinated flowers might have shaped colour preferences or learning speed in some stingless bees; stingless bees, however, have evolved very effective recruitment to novel food sources (Jarau et al. 2003), that might compensate lacking mechanisms to find inconspicuous food sources.

Colour hue preference

Several studies could show preferences for colour stimuli with a dominant wavelength in the blue wavelength band in bumblebees and honeybees (Menzel 1967; Giurfa et al. 1995; Gumbert 2000; Dyer et al. 2007; Ings et al. 2009; Hudon and Plowright 2011; Morawetz et al. 2013) and also in stingless bees (Dyer et al. 2016). The results of our study do not fit these findings, since *M. quadrifasciata* showed a distinct preference for the bee-green colour and *M. mondury* preferred the bee-UV-blue over the bee-green colour, but largely ignored the bee-blue colour. Since the colony size of *M. mondury* is much higher (3000–10,000 individuals) than in *M. quadrifasciata* colonies (300–500 individuals) the individual success of a worker is not that important for the survival of the colony (Hubbell and Johnson 1977; Viana et al. 2015). This reduced pressure could allow *M. mondury* workers to switch to flowers which do not fit to their innate preferences and thus foraging on the level of single worker bees become more flexible. Given the growing interest in stingless bees for plant pollination (Heard 1994, 1999; Heard and Dollin 1998; Bispo dos Santos et al. 2009; Hikawa and Myanaga 2009; Potts et al. 2010), resolving these questions with additional experiments will be of high value for understanding how to best use stingless bees for crop pollination management.

Conclusions

A previous study testing the preferences of bumblebees and honeybees in regard to the pigment concentration of inked filter paper by Papiorek et al. (2013) found clear evidence that the pigment concentration did not predict the bees' preference rather than the perceived spectral purity. The results of this study provide evidence that it is not a certain combination or interaction of colour intensity and spectral purity, which mediates the decisions of the bees. The stingless bees did not choose colours solely based on high spectral purity, but colour intensity seems an additional factor determining the bees' colour choice if colours display low spectral purity. The results do not exclude an interaction of colour intensity and spectral purity when the spectral purity is at a medium level, while bees seemingly did not consider intensity when spectral purity is at a high level. Interestingly, recent colour preference testing on stingless bees in Australia also suggests a complex interaction of colour traits contributing to colour choices by *Tetragonula carbonaria* bees (Dyer et al. 2016). Spectral purity and colour intensity are largely considered interdependent colour parameters at least in human colour vision (Luebbe 2013).

To humans darker colours are perceived more saturated than brighter colours of same colourfulness.

M. mondury and *M. quadrifasciata* workers are about the size of honeybee workers (Kerr and Nielsen 1966) and both species are considered for pollination management of crops (Heard 1999; Hikawa and Myanaga 2009; Bispo dos Santos et al. 2009). It is, however, currently not known whether these species have foraging specialities in tropical rainforests in regard to light environment that might have shaped their preferential colour choice. In future experiments an analysis of innate colour preferences of naïve *M. mondury* and *M. quadrifasciata* workers will be tested to reveal if the colour preferences found in this study reflect ecologically relevant differences between these two closely related species, and to what extent such preferences may be influenced by exposure to natural food plants.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no competing interests.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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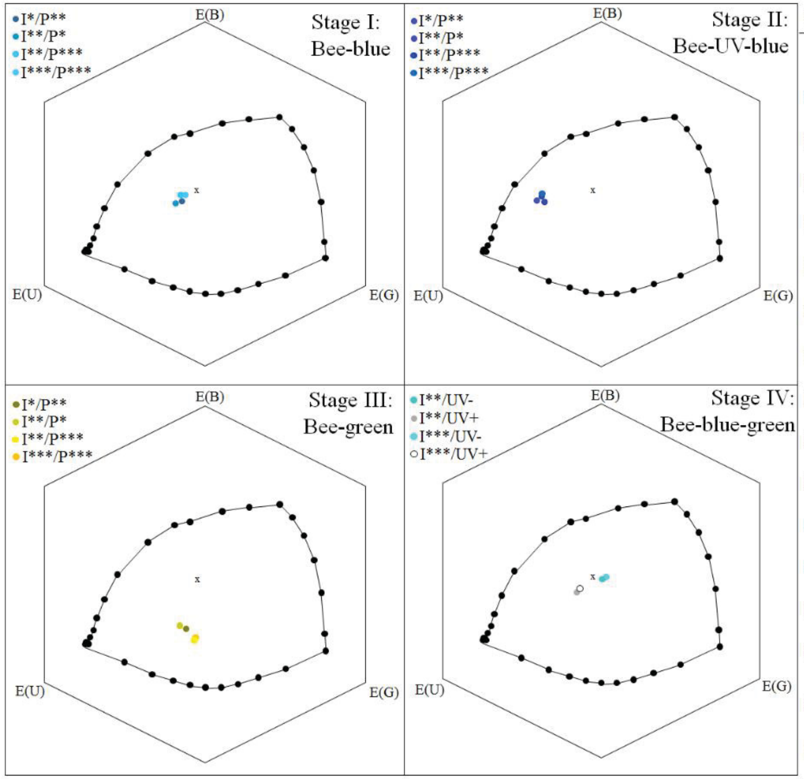
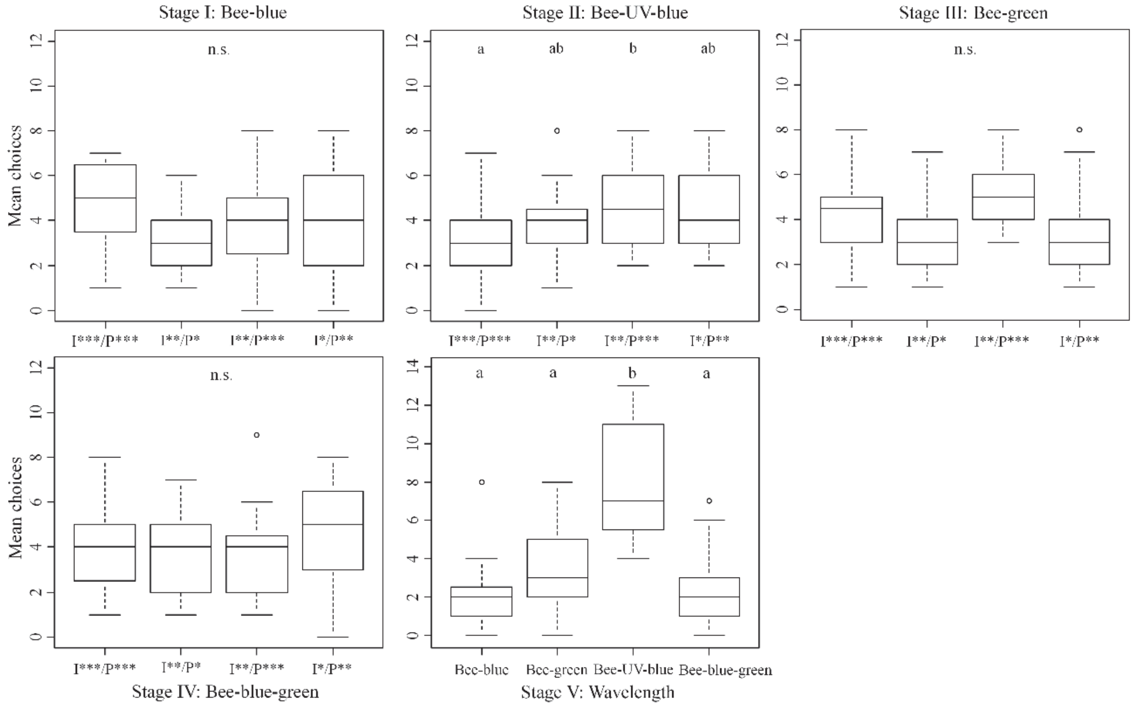
Supplement

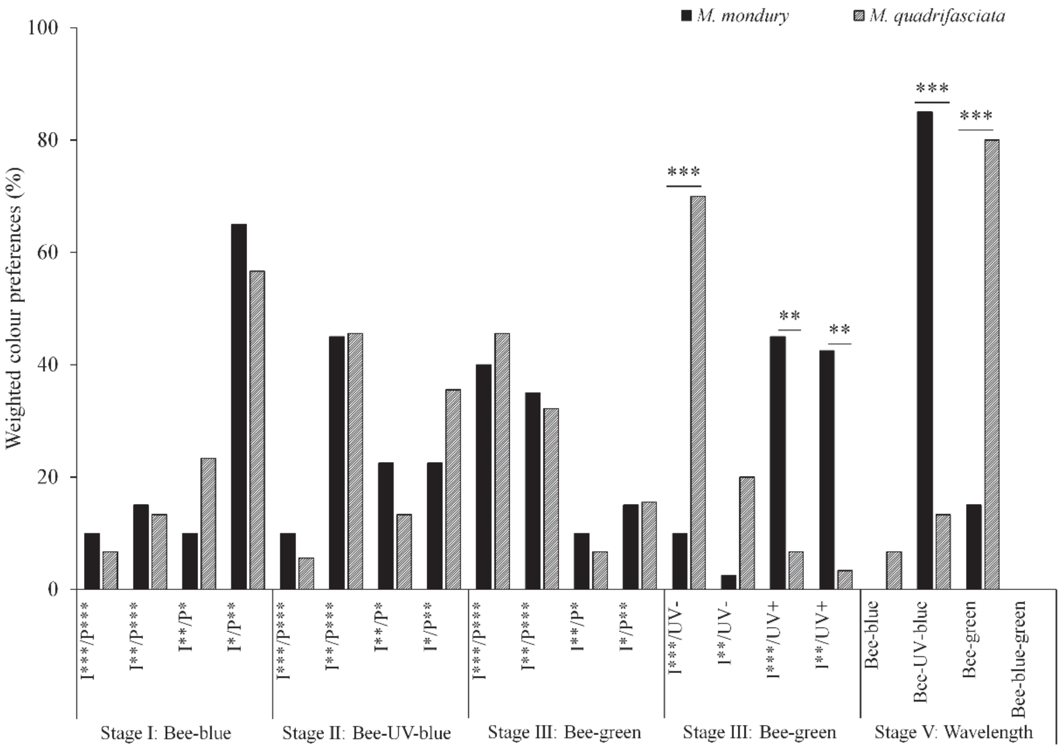
Online Resource 1 Colour loci of the test stimuli of stage I-IV with green background in the colour hexagon (Chittka 1992) with calculated values of the receptor-specific contrast, chromatic contrast, spectral purity and green contrast.



Stimulus	Receptor specific contrast			Chromatic contrast	Spectral purity	Green contrast
	S	M	L			
Bee-blue I***/P***	0.78	0.71	0.65	0.11	0.22	0.15
Bee-blue I**/P***	0.73	0.59	0.55	0.12	0.23	0.05
Bee-blue I**/P*	0.71	0.63	0.58	0.16	0.27	0.08
Bee-blue I*/P**	0.60	0.49	0.45	0.14	0.23	-0.05
Bee-UV-blue I***/P***	0.81	0.61	0.43	0.32	0.65	-0.07
Bee-UV-blue I**/P***	0.77	0.54	0.41	0.33	0.64	-0.09
Bee-UV-blue I**/P*	0.77	0.56	0.39	0.31	0.57	-0.11
Bee-UV-blue I*/P**	0.73	0.49	0.34	0.34	0.54	-0.16
Bee-green I***/P***	0.72	0.37	0.66	0.32	0.55	0.16
Bee-green I**/P***	0.72	0.40	0.57	0.33	0.56	0.07
Bee-green I**/P*	0.65	0.29	0.58	0.28	0.47	0.08
Bee-green I*/P**	0.56	0.25	0.45	0.27	0.47	.0.05
Bee-blue-green I***/UV-	0.75	0.78	0.78	0.03	0.04	0.28
Bee-blue-green I**/UV-	0.73	0.75	0.75	0.01	0.03	0.28
Bee-blue-green I***/UV+	0.91	0.76	0.75	0.13	0.20	0.25
Bee-blue-green I***/UV+	0.92	0.79	0.78	0.15	0.22	0.25



Online Resource 2 Colour preferences of *Melipona mondury* tested with colour stimuli presented against green background. The colour choices of the tested bees (n = 20) are plotted against the tested stimuli for every test stage. Workers were tested for bee-blue (stage I), bee-UV-blue (stage II), bee-green (stage III), and bee-blue-green (stage IV) colours. Subsequently the workers were tested for the most preferred colour hue (stage V). Each stage comprised four colours of the same dominant wavelength which varied in colour intensity and colour purity to test the impact of these two colour traits on the bees' colour choice. I = colour intensity, P = colour purity; * = low, ** = medium, *** = high; UV- = ultraviolet-absorbing; UV + = ultraviolet-reflecting. Different letters indicate significant differences, n.s. = not significant (Tukey all-pair comparisons)



Online Resource 3 Weighted colour preferences of individual bees of *M. mondury* (black bars) and *M. quadrifasciata* (shaded bars) for the five tested stages. The mean choice frequency of each individual bee were weighted with the most frequently visited stimulus scored 1 and the other three stimuli scored 0. I = colour intensity, P = colour purity; * = low, ** = medium, *** = high; UV- = ultraviolet-absorbing; UV + = ultraviolet-reflecting; the symbols above the columns represent the statistical analysis by Fisher's exact test: *** = $p < 0.001$, ** = $p < 0.01$

CHAPTER 4

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Spectral purity, intensity and dominant wavelength: Disparate colour preferences of two Brazilian stingless bee species

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Abstract

Bees use floral colour as a major long distance orientation cue. While it is known for bumblebees and honeybees that dominant wavelength (≙ colour hue), colour contrast and spectral purity (≙ saturation) are crucial for flower detection and discrimination, only little is known about colour preferences in stingless bees (Meliponini). In this experiment freely flying workers of two Brazilian species of stingless bees – *Partamona helleri* and *Melipona bicolor* – were tested for colour preferences concerning the colour parameters dominant wavelength, spectral purity and intensity (≙ brightness). Each individual bee had to perform 57 tests, in which a definite series of dual choices between colour stimuli varying in intensity, spectral purity or dominant wavelength were presented. The results show that *P. helleri* chose colours of higher spectral purity and preferred bluish colours, while *M. bicolor* made generalized colour choices. Intensity did not influence the colour choice of any bee species. The results of *P. helleri* are consistent with findings for honeybees and bumblebees, while colour preferences in *M. bicolor* seem to be absent.

Introduction

Stingless bees are considered important pollinators in tropical and subtropical regions. Stingless bees are the most speciose, the most abundant and most diverse group of eusocial bees [1]. The human food consumption worldwide causes the demand for pollination management with native or introduced bees in tropical regions. Introduced bee species like honeybees endanger native species and can lead to extinction of local populations [2-4]. Unlike honeybees only few studies investigate stingless bees and their value for crop pollination [5,6]. Honeybees are the preferred bee species for crop pollination although many stingless bees show comparable characteristics [7]. Stingless bees do not use a dance language like honeybees to share information concerning food sources, but use trophallaxis, excited movements, sound production, body contact, odour traits, chemical markings or visual tracking of nestmates to share information [8-9]. Furthermore, queens of stingless bees are replaced by their offspring leading to a long lifespan for colonies [10,11]. Despite many common features, stingless bees are much more diverse than honeybees (e.g. body size, foraging strategy, and colony size) and are able to provide pollination services that honeybees may not be able to provide (e.g. buzz pollination of flowers with poricidal anthers by *Melipona* see Sarto et al. [12]). The lack of a functional sting is an additional advantage in particular for enclosed places like green houses or urban areas. Most stingless bees forage in high-density groups on food sources that were located by scout bees [13,14]. High-density foragers can be divided into two groups – non-aggressive foragers, including e.g. *Melipona*, *Partamona* and *Scaptotrigona,* and aggressive foragers like *Trigona* [15]. Aggressive foragers have less scout bees than non-aggressive ones but drive away non-aggressive bees from located food sources [15]. To avoid the loss of a food source, non-aggressive foragers need to exploit their food sources quickly before they are detected by aggressive foragers [15]. The evolutionary pressure to find food sources leads to the question how stingless bees detect flowers. For honeybees and bumblebees many studies confirmed the importance of floral colour for the detection of flowers [16-19]. So far only very few studies analysed colour perception in stingless bees [20,21, 22-23]. Generally, bees possess three photoreceptor types with maxima at ~340nm 'UV', ~430nm 'blue' and ~540nm 'green' [16, 24-25]. The distribution of photoreceptors can be found among all genera of bees and suggests phylogenetic constraints for bee vision [25-26]. Studies about colour perception in bees have identified important traits of colours that facilitate detection, recognition and discrimination of colours and thus aid colour choice in bees. The main traits of colours that influence bees are dominant wavelength (≙ hue), spectral purity (≙ saturation) and green contrast, while the colour intensity (≙ brightness) is discussed to have no influence on bees' colour choice [17-19,27-31].

In general, bees appear to have a preference for blue colours, but also preferences for yellow in bumblebees and for UV-absorbing white colours in stingless bees could be observed [17,20,21,28,32]. The chromatic perception of bees depends on the visual angle between bee and target. If the visual angle is below 15° honeybees only perceive colours with their green receptor – also known as green contrast [17]. If the visual angle surpasses 15° honeybees are able to use colour vision [17]. In bumblebees a visual angle of 2.7° is sufficient to perceive colours [19]. The green contrast functions for far-distance detection of flowers, while chromatic contrast functions for close-distance recognition and both are important for flower detection in bees [18,19,33]. The contrast of a colour against the background is an important cue for bees and influences the choice behaviour of bees [17,34]. The size of a target can influence whether bumblebees use green or colour contrast to detect flowers and honeybees' decisions concerning target shape are influenced by the background colour [18,35]. A study conducted by Spaethe and colleagues [36] found that the discrimination of colours is poorer in *Trigona* cf. *fuscipennis* and *Tetragonula carbonaria* than in honeybees and bumblebees. Furthermore, spectral purity of colours influences the choice of honeybees and bumblebees. When bumblebees and honeybees have to choose between stimuli of the same dominant wavelength but with different values of spectral purity the stimuli with higher spectral purity are preferred over less spectrally pure stimuli [29,30]. These results could not be verified for stingless bees so far [20,21]. Unlike spectral purity, the intensity of colours is assumed to have no influence on bees' colour choice [31,37,38]. Consequently, colour vision models like the colour hexagon by Chittka [37] take no account of intensity. However, Hempel de Ibarra et al. [39] found, based on experimental data, that an increase of intensity in light stimuli improves colour discrimination of honeybees probably based on contrast between floral colour and background.

In this study, colour choices of two stingless bee species, *Melipona bicolor* and *Partamona helleri* (both belong to the tribe Meliponini) were analysed. The aim is to see whether these two stingless bee species share similar preferences known for honeybees or bumblebees. Do these two stingless bee species prefer specific dominant wavelengths, like honeybees and bumblebees are known to prefer blue colours, and is their choice also depending on spectral purity? Observations in the field showed that many stingless bees forage on red bird-pollinated flowers, although these flowers appear achromatic to bees [40-43]. This might be explained by the use of intensity cues or green contrast for flower recognition in stingless bees. Therefore, we tested freely flying workers of the two stingless bee species following a short training to the test area in a series of dual choice tests in which distinct colour parameters were varied.

Material and methods

Production and characteristics of stimuli

The colour stimuli based on a variety of basic colour pigments (Artist Pigments: “Sky Blue”, “Ultramarine Blue”, “Yellow”, “Bright Red Ochre” and “Zinc White”, Art Material International Warenhandelsgesellschaft mbH, Kaltenkirchen, Germany) that were mixed with achromatic pigments (black = “Dei®Art Russverkollerung”, Deifel GmbH & Co. KG, Schweinfurt, Germany; white = Barium sulphate, 98% extra pure, Acros Organics BVBA, Geel, Belgium; grey = defined mixture of white and black, see S1 Table). The resulting colours were measured via spectrometer analysis (USB4000 miniature fibre optic spectrometer, Ocean Optics GmbH, Ostfildern, Germany) at an angle of 45° using a UV-NIR deuterium halogen lamp (DH-2000-BAL, Ocean Optics GmbH), which was connected to the spectrometer by a UV–VIS fibre optic cable (Ø 600 µm, QR600-7-UV 125 BX, Ocean Optics GmbH). The obtained spectral data were plotted in the colour hexagon by Chittka [37] (see Fig 1). The receptor-specific contrast (q_i) between stimulus and background is calculated based on the quantum flux (Q_i) given by:

$$Q_i = \int_{300}^{700} S_i(\lambda)\mathrm{I}(\lambda)\mathrm{D}(\lambda)\mathrm{d}(\lambda)$$

$$q_i = \frac{Q_i(stimulus)}{Q_i(background)}$$

where $S_i(\lambda)$ refers to the spectral sensitivity function of the photoreceptor type *i* (UV, blue and green) considering the spectral sensitivity of *M. quadrifasciata.* $D(\lambda)$ is the illumination (here D65 standard illumination) and $d(\lambda)$ denotes the wavelength step size [16].

Based on these results the amount of light absorbed by each photoreceptor type is given by:

$$P = Q_i * R$$

where *R* is the sensitivity factor simulating the adaptation of the photoreceptor types to the background (I_B):

$$R = 1/\int_{300}^{700} S_i(\lambda) I_B(\lambda) D(\lambda) d(\lambda)$$

The absorption of each photoreceptor (P) can be transduced into photoreceptor excitation (E) by:

$$E = P/(P+1)$$

For further analysis of the bees' choice behaviour, the chromatic contrast was calculated according to the colour hexagon by Chittka [37]. It is defined as the perceptual distance between a colour locus and the background given in hexagon units. The spectral purity results from the perceptual distance between a colour locus and the background in relation to the distance between the background and the spectral line [33].

$$SP = \frac{H_i(target - background)}{H_i(spectral\ locus - background)}$$

The intensity was calculated by adding up the values of the receptor excitation for all three photoreceptors and dividing those by three [18]. Based on the results of these calculations, four stimuli of each dominant wavelength were selected (S1 Table). In addition to the calculations according to the hexagon, the values for saturation and luminance were calculated according to Valido et al. [44] which are based on the reflectance of stimuli and does not include the photoreceptor sensitivities of the receiver (S1 Table). The pigments were compacted into culture dishes (35 mm diameter, 10 mm height) by using a mechanical press (custom made).

The experimental setup

For the experimental setup, two PVC panels (50 cm x 50 cm; 50 cm x 25 cm) were connected with a hinge (S1 Figure). The smaller PVC panel was used as a base to stabilise the bigger PVC panel that was fixated at an angle of 45°. A metal plate attached to both PVC panels stabilised the structure. The bigger PVC panel was covered with a grey Styrofoam wallpaper reflecting constantly throughout the UV and visible range of wavelength (Climapor® Insulation Wallpaper Graphite Laminated with Pasteboard, Saarpor Klaus Eckhardt GmbH Neunkirch
en Kunststoffe KG, Neunkirchen, Germany). Two petri dish lids were affixed to the wallpaper, using Velcro® tape, with 5 cm distance to the midpoint of the PVC sheet and functioning as receptacles for the pressed colour stimuli. Below each stimulus a balcony made of metal plate covered with Styrofoam wallpaper was affixed as a landing platform for the bees holding a PCR tube lid in the centre to offer sucrose solution to the bees.

Bee keeping and conditioning

The hives of *Melipona bicolor* and *Partamona helleri* were located at the campus of Universidade de São Paulo (USP) in the garden of the BeeLab. The nest of *M. bicolor* was placed inside the lab with an entrance leading outside while the nest of *P. helleri* was located outside of the lab. The workers of both species were freely flying and flower experienced. Gravity feeders with ~10-30% sucrose solution were placed in close proximity to hives of a variety of stingless bees. Most species (*Melipona quadrifasciata*, *Scaptotrigona depilis* and *Trigona spinipes*) were deterred by honeybee workers and only workers of *P. helleri* were voluntarily feeding at the feeder. For the training of *P. helleri*, workers were caught at the feeder and then trained to forage at the experimental setup. Each worker was labelled with nail polish to identify individuals. Workers of *M. bicolor* were trained individually from the entrance of their nest to the experimental setup by leading the way with sucrose solution. Since no recruitment by the bees happened, each worker had to be trained individually and could be tested as such. In total, 24 individuals of *P. helleri* and 20 individuals of *M. bicolor* were tested.

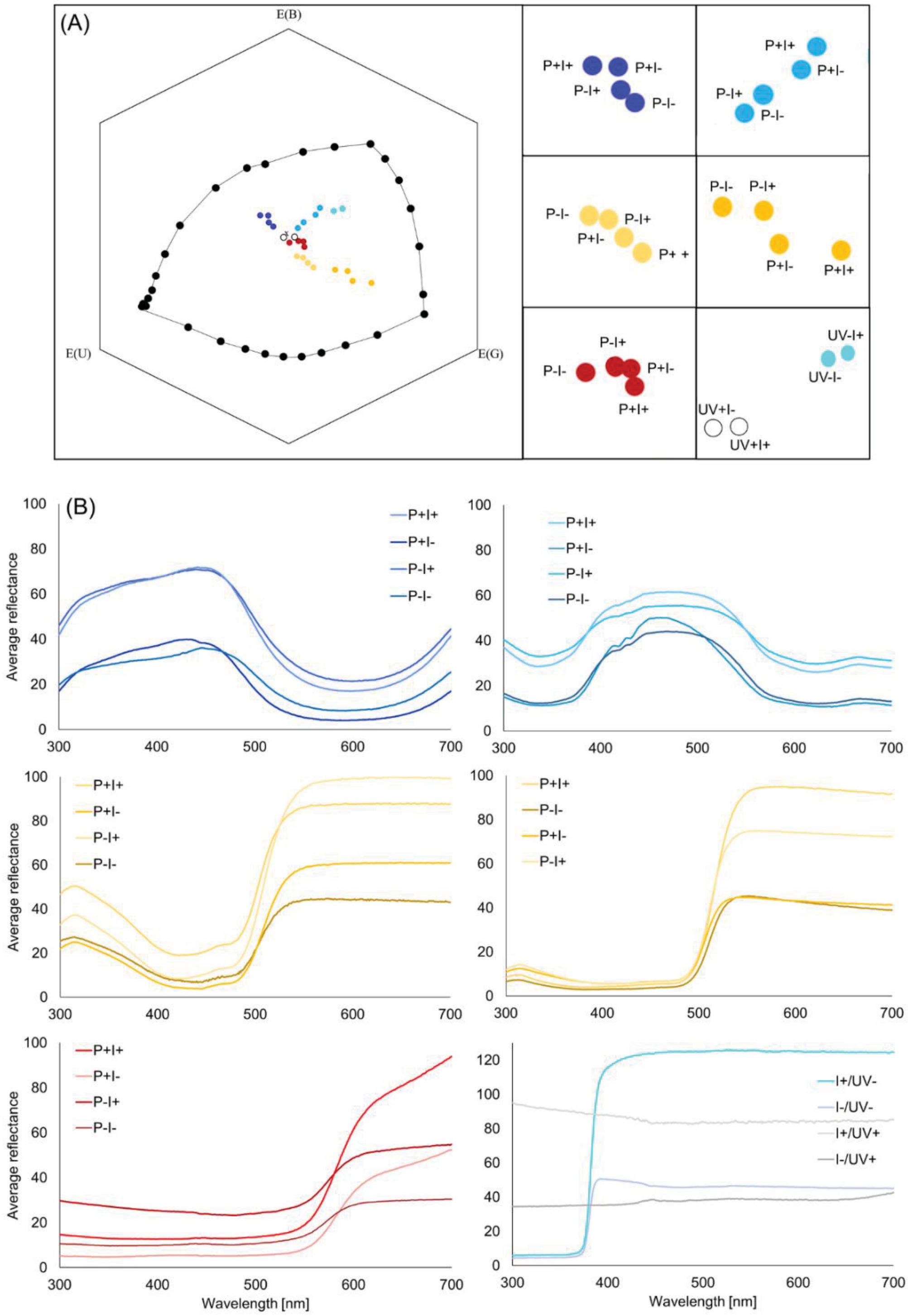

Fig 1. Analysis of used colour stimuli.

(A) The colour hexagon according to Chittka [37] displays the perception of colours in accordance with bee-specific photoreceptor sensitivities (*Melipona quadrifasciata*), the background (grey Styrofoam wallpaper) and the ambient light (standard daylight illumination D65) (from top right to bottom left: UV-blue, blue, UV-yellow, yellow, red, white) [16]. (B) Reflectance curves of all colour stimuli that are included in the six colour categories used in the experiments (from top right to bottom left: UV-blue, blue, UV-yellow, yellow, red, white).

The experimental procedure

Prior to the experiment, the bees were trained to visit both balconies of the experimental setup to avoid any effect of the stimuli’s position. During the training no stimuli were offered, only the empty petri dish lids were presented. After a bee had flown several times to both balconies, the experiment started.

A total of 57 definite dual choice tests were offered in a semi-randomised order (see S2 Table) to the bees in which all four stimuli of one colour category were tested against each other (6 tests per colour category, 36 in total) and the seven dominant wavelengths (most intense and spectrally purest stimulus of UV-blue, blue, UV-yellow, yellow, UV-reflecting white, UV-absorbing white and red) were tested against each other (21 tests in total).

The colour categories were mixed in its order and the tests within one colour category were not conducted consecutively. To avoid conditioning caused by the order of tested stimuli, the order was turned around for some of the bees. Each bee made one decision per foraging bout. While the bee returned to the hive the stimuli were changed and the reward refilled.

Statistical analysis

The statistical program R was used to analyse the data [45]. All data were tested for normal distribution by using the Shapiro-Wilk test.

The pooled data were analysed by testing the bees’ choices for the different stimuli of each colour category using a generalised linear mixed model (GLMM) [46]. We used the “lme4” package of R [47] to analyse the individual choices of the bees, which were assessed using GLMM with binomial distribution of data and the best linear fit depending on akaike information criterion (AIC) score. For the overall test, we analysed the number of choices for each stimulus as fixed effect and each individual bee was given a number. This numbering was used as random effect of the model. To test the distribution of choices between the four stimuli of each stage, a multiple comparison of means was done with the Tukey all-pair comparisons.

For the comparison of two-data samples, the Student’s *t*-test or the Mann-Whitney-*U* test were used.

Results

Statistical analysis of colour choice behaviour for *Melipona bicolor* and *Partamona helleri* reveals that both species chose colours differently. Workers of *M. bicolor* do not show clear preferences within any of the tested colour categories (Fig 2). Only for the white colour category the UV-absorbing stimulus with reduced intensity was preferred by *M. bicolor* as well as the more intense stimuli in UV-yellow category. Concerning dominant wavelength, workers of *M. bicolor* showed no distinct preferences (Fig 3).

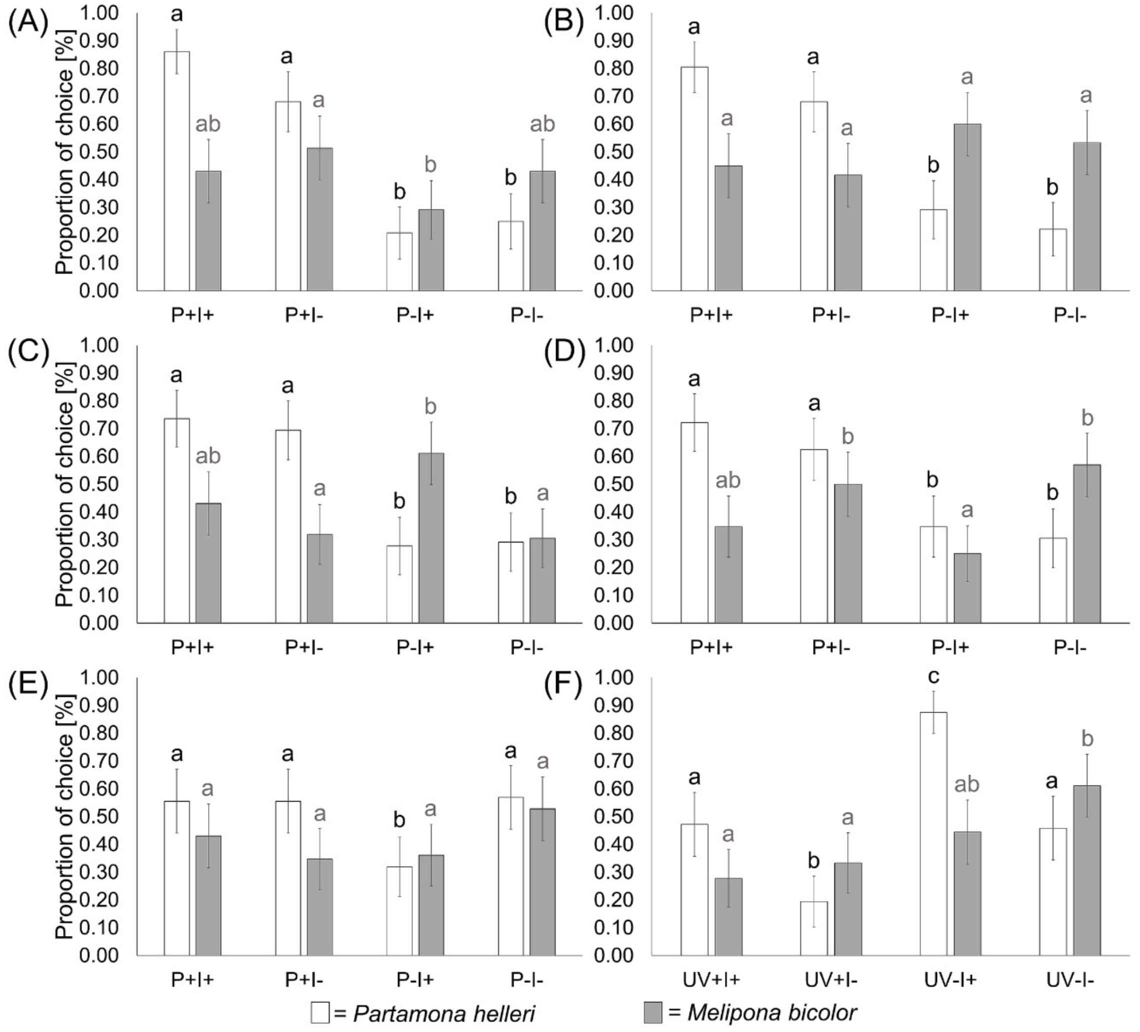

Fig 2. Colour choices within the colour categories.

Six categories of colours were tested (A) = UV-blue, (B) = blue, (C) = UV-yellow, (D) = yellow, (E) = red and (F) = white. Each colour category consists of four stimuli with different levels of spectral purity (P+ = high spectral purity; P- = low spectral purity) and colour intensity (I+ = high colour intensity; I- = low colour intensity). Only in the white colour category spectral purity is replaced by UV properties of colours (UV+ = UV-reflecting; UV- = UV-absorbing). The total choices of *Partamona helleri* (black columns; n = 24) and *Melipona bicolor* (grey columns, n = 20) were compared by using a GLMM with Tukey's all pair comparisons as post-hoc test. Different letters above the columns show statistical significances, where the same letters represent no significant results and different letters represent significant results. Error bars indicate binomial confidence intervals.

Workers of *P. helleri* showed strong differences in their choice behaviour compared to workers of *M. bicolor* (Fig 2). The stimuli with high spectral purity were generally preferred over less spectrally pure stimuli in the UV-blue, blue, UV-yellow and yellow colour category. In the red colour category, no preference for any of the stimuli could be observed. Furthermore, workers of *P. helleri* preferred UV-absorbing white colours over UV-reflecting white ones and also preferred UV-absorbing white and blue stimuli (both stimuli were chosen in 108 of 144 executed dual choice tests, n = 24, 6 dual choices per colour) over the other dominant wavelengths (Fig 3). Red was chosen least compared to the other dominant wavelengths (only chosen in 17 of 144 executed dual choice tests, n = 24, 6 dual choices per colour).

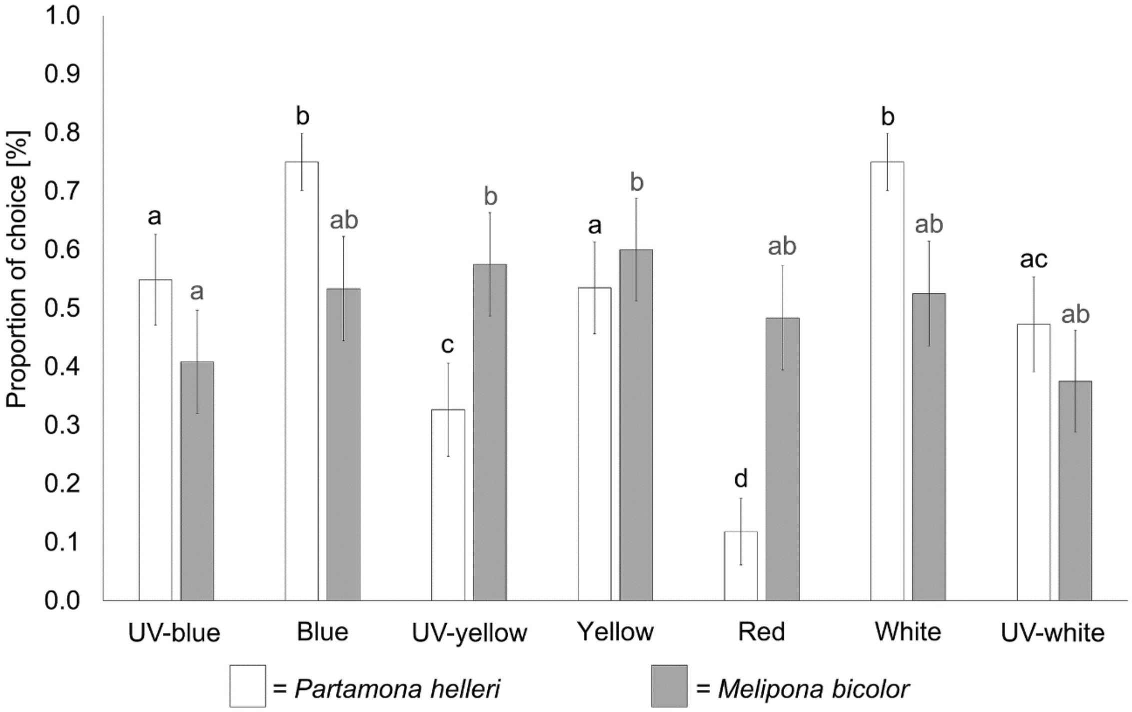

Fig 3. Colour choices according to differences in dominant wavelength. Seven dominant wavelengths (UV-blue, blue, UV-yellow, yellow, red, white and UV-white) were tested in dual choice experiments (each individual choose 21 times). The total number of choices of *Partamona helleri* (black columns; n = 24) and *Melipona bicolor* (grey columns, n = 20) were compared by using a GLMM with Tukey's all pair comparisons as post-hoc test. Different letters above the columns

show statistical significances, where the same letters represent no significant results and different letters represent significant results. Error bars indicate binomial confidence intervals.

To analyse the effect of spectral purity and colour intensity on the colour choice behaviour of both stingless bee species, the total choices of spectrally purer colours (P+) were compared to the total choices for less spectrally pure colours (P-) and the total number of choices for more intense colours (I+) against the total number of less intense colours (I-) without considering dominant wavelength (Fig 4). None of the tested parameters elicit a distinct colour choice in *M. bicolor* (spectral purity: *t* = -0.6589, df = 9, p = 0.5264; intensity: *t* = -0.8655, df = 11, p = 0.4053, Student's *t*-test) while *P. helleri* chose spectrally purer colours significantly more often than less pure colours (W = 98, p = 0.0003, Wilcox test) but do not pick colours based on intensity (W = 93, p = 0.236, Wilcox-test).

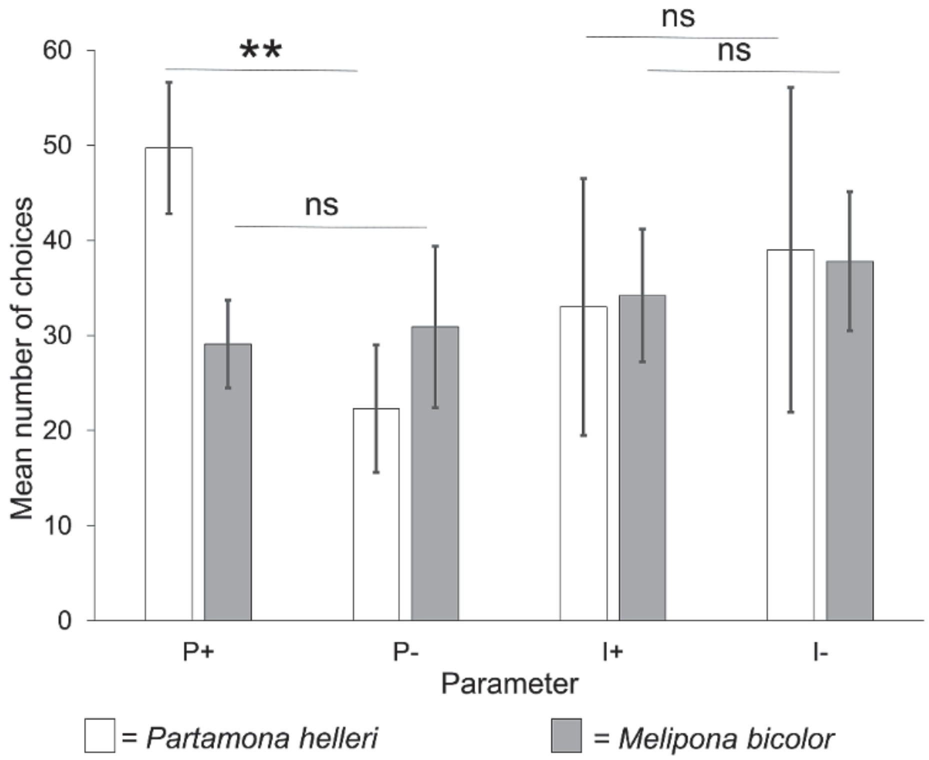

Fig 4. Mean number of choices according to spectral purity or intensity independent of dominant wavelength.
The level of high spectral purity (P+) differs significantly when compared to the low spectral purity level (P-) for *Partamona helleri* (n = 24). For stimuli that differ in colour intensity the choices reveal no significant preferences for neither *P. helleri* nor *Melipona bicolor* (n = 20).

Discussion

In the current study, we observed a preference for spectrally purer colours for workers of *Partamona helleri,* while *Melipona bicolor* generalized colours independent of dominant wavelength, intensity and spectral purity. In a previous study, *Melipona mondury* and *Melipona quadrifasciata* were tested concerning their colour preferences and similar results were obtained [21]. Both *Melipona* species chose colours independently of intensity and spectral purity and only minor preferences for UV-blue (*M. mondury*) or yellow (*M. quadrifasciata*) could be obtained.

Floral colour is one of the strongest advertisements by flowering plants and constitutes a long distance effect of flowers on flower visitors. In order to locate flowers bees need specific mechanisms to detect and recognize colours to collect food rewards most effectively. In honeybees and bumblebees dominant wavelength (≙ colour hue) and spectral purity (≙ saturation) were identified as main colour parameters influencing honeybees' and bumblebees' choice as well as colour contrast to the background and green contrast [17], [18], [19], [28], [29], [30]. So far, little is known about colour preference in stingless bees. Dyer et al. [20] found a preference for UV-absorbing white colours in *Tetragonula carbonaria*, but more specific data concerning a preference for specific colour parameters like spectral purity or intensity (≙ brightness) could not be found yet [21].

One possible reason for the differences in the spontaneous colour choice between *M. bicolor* and *P. helleri* could be the recruitment behaviour of workers in these two species. Stingless bees are known to use chemical communication and chemical marking to exploit food sources [47]. Especially for high-density foragers, like *Melipona* and *Partamona*, chemical communication is important to recruit nest mates to the direction where rewarding food sources are located and the position of a food source itself. Naïve workers can either act as scout bees or as recruits that are informed by other scout bees [15]. In the experiments, the stimuli presented in a test were cleaned after each use so that chemical communication via scent-marked stimuli should not influence the experimental outcome. Each worker of *M. bicolor* had to be trained individually to the test arena because the tested workers did not recruit other workers, while *P. helleri* workers were frequently recruited by tested bees. The missing recruitment of *M. bicolor* could be explained by the small distance between hive and test arena (approximately 1.5 m). Species of the genus *Melipona* mark their food sources directly but do not place chemical markings along the way to a food source and a short way like 1.5 m could be insufficient to guide other workers from the nest entrance to the food source [48].

Another reason for the dissimilarity between the colour choices of the two bee species could be the different size of the colonies. *P. helleri* hives harbour up to 10000 individuals (personal communication Sergio Dias Hilário, USP), while *M. bicolor* hives only harbour up to 1000 individuals [49], [50]. This difference in number could raise the pressure on *M. bicolor* being more generalistic than *P. helleri*.

The observed preference for spectrally purer colours in *P. helleri* accords to results observed in flower-experienced honeybees and bumblebees where workers spontaneously preferred spectrally purer colours of the same dominant wavelength independent of their conditioning [29], [30]. A field study in Greece showing a correlation between the amount of produced nectar and the spectral purity values of floral colours suggests that a preference for spectrally purer colours by bees could be advantageous to find higher rewarding flowers [51]. The choices concerning dominant wavelength of *P. helleri* assort to known preferences in honeybees, bumblebees and Australian stingless bees which showed preferences for bluish colours [20], [28], [32].

The calculated values for intensity (bee-subjective vision) and luminance (physical values) are in accordance with each other, while the values for spectral purity (bee-subjective vision) and saturation (physical values) only resemble each other for the red stimuli (see S1 Table). While the obtained results *for P. helleri* support the bee-subjective values calculated with the hexagon model by Chittka [37] the results obtained for *M. bicolor* can be explained with neither physical nor bee-subjective calculations. Based on the choices of *P. helleri* that can only be explained by the colour hexagon this model appears to be a solid method for the calculation of spectral purity.

Many studies analysed colour choice in hummingbirds and found that experienced hummingbirds showed preferences for red colours but naïve hummingbirds show no spontaneous preferences for specific colours and instead rather decide for location or quality of a food source [52], [53], [54], [55], [56], [57], [58]. Furthermore, a study by Lunau et al. [59] observed the absence of colour preferences in hummingbirds for UV-absorbing red and UV-reflecting white flowers, though these are typical floral colours of hummingbird pollinated flowers [60], [61], [62]. The results of that study suggest that hummingbirds engage a private niche that is created by the inability of other pollinators (in this case orchid bees) to detect these floral colours. This "bee avoidance" hypothesis has been confirmed in the field by Bergamo et al. [63]. So far, all experimental testing of colour preferences in the genus *Melipona* (three species *M. bicolor*, *M. mondury* and *M. quadrifasciata*) could only show slight preferences for specific colours with no pervading pattern [21]. In this view, *Melipona* developed different mechanisms to locate food sources other than colour perception and is thus less excluded by flower colours of low spectral purity that specifically allure hummingbirds, i.e. UV-reflecting white and UV-absorbing red.

In total, these results show that a generalization of colour preferences in bees is misleading since *M. bicolor* and *P. helleri* show strong differences in their colour choices. *M. bicolor* shows no colour choice behaviour, while *P. helleri* shows a similar colour choice behaviour in comparison to honeybees and bumblebees. Flower detection in *Melipona* seems to be less dependent on colour vision than on other criteria like chemical marking, odour or location of food sources.

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Supplement

S1 Figure. Experimental setup.

S1 Table. Mixture ratios of colour pigments and calculated colour parameters of compacted stimuli.

(Black* = see stimulus Black; Grey* = see mixture Grey; Yellow-grey* = see mixture Yellow-grey; P+ = high spectral purity; P- = low spectral purity; I+ = high intensity; I- = low intensity; UV- = UV-absorbing; UV+ = UV-reflecting).

Pigment mixtures for 10g	Mixture name	Dominant wavelength	Intensity	Spectral purity	Green contrast	Luminance/1000	Saturation
„Ultramarine Blue“(1.0g)+Mixture for UV-blue P-I+ (9.0g)	UV-blue P+I+	409	0.62	0.40	0.05	17.52	0.63
„Ultramarine Blue“(10.0g)	UV-blue P+I-	409	0.45	0.46	-0.16	7.99	0.90
„Ultramarine Blue“(3.0g)+Barium sulfate(5.0g)+Grey(2.0g)	UV-blue P-I+	407	0.63	0.27	0.07	18.56	0.54
„Ultramarine Blue“(8.0g)+Barium sulfate(1.5g)+Black*(0.5g)	UV-blue P-I-	410	0.45	0.29	-0.12	8.72	0.64
Mixture for Blue P-I+(8.0g)+Barium sulfate(1.95g)+Black*(0.05g)	Blue P+I+	474	0.58	0.30	0.10	16.58	0.43
„Sky Blue“(10.0g)	Blue P+I-	474	0.46	0.33	0.00	9.49	0.83
„Sky Blue“(5.0g)+Grey*(5.0g)	Blue P-I+	474	0.58	0.19	0.09	16.62	0.31
„Sky Blue“(9.95g)+Black*(0.05g)	Blue P-I-	478	0.46	0.20	0.00	9.64	0.67
„Buttercup Yellow“(2.708g)+Black*(0.0056g)+Barium sulfate(7.28625g)	UV-yellow P+I+	554	0.58	0.28	0.19	23.21	0.60
Yellow-grey*(8.5g)+Barium sulfate(0.5g)+“Buttercup Yellow“(1.0g)	UV-yellow P+I-	555	0.52	0.26	0.12	13.83	0.83
Yellow-grey*(9.5g)+Barium sulfate(0.25g)+“Buttercup Yellow“(0.25g)	UV-yellow P-I+	560	0.63	0.17	0.20	22.53	0.82
„Buttercup Yellow“(2.475g)+Barium sulfate(7.425g)+Black*(0.1g)	UV-yellow P-I-	565	0.52	0.15	0.08	11.47	0.67
„Buttercup Yellow“(9.95g)+Black*(0.05g)	Yellow P+I+	530	0.29	0.61	0.09	18.42	0.99
Mixture for Yellow P-I-(5.0g)+Barium sulfate(5.0g)	Yellow P+I-	533	0.27	0.60	-0.05	9.94	0.79
„Buttercup Yellow“(5.0g)+Barium sulfate(4.95g)+Black*(0.05g)	Yellow P-I+	531	0.31	0.43	0.05	15.61	0.89
„Buttercup Yellow“(9.5g)+Black*(0.5g)	Yellow P-I-	530	0.21	0.43	-0.07	8.95	0.95
„Bright Red Ochre“(2.0g)+Barium sulfate(8.0g)	Red P+I+	533	0.31	0.13	-0.14	13.37	1.22
„Bright Red Ochre“(8.0g)+Barium sulfate(2.0g)	Red P+I-	522	0.16	0.12	-0.29	6.78	1.41
Mixture for Red P+I+(2.0g)+Barium sulfate(4.0g)+Grey*(4.0g)	Red P-I+	603	0.44	0.05	-0.05	13.68	0.46
Mixture for Red P+I+(9.5g)+Black*(0.5g)	Red P-I-	522	0.25	0.06	-0.21	6.67	0.62
Barium sulfate(10.0g)	UV-white I+	381	0.73	0.03	0.22	39.85	-
Barium sulfate(9.83g)+Black*(0.17g)	UV-white I-	505	0.52	0.01	0.03	15.25	-
„Zinc White“(10.0g)	White I+	488	0.68	0.48	0.28	34.42	-
„Zinc White“(9.75g)+Black*(0.25g)	White I-	487	0.50	0.28	0.08	15.01	-
Barium sulfate(9.7436g)+Black*(0.2564g)	Grey	-	-	-	-	-	-
„Buttercup Yellow“(1.25g)+Barium sulfate(8.6875g)+Black*(0.0625g)	Yellow-grey	-	-	-	-	-	-
„Rußverkollerung“(10.0g)	Black	-	-	-	-	-	-

S2 Table. Semi-randomized order of dual choice tests.

(UVB = UV-blue; B = blue; UVY = UV-yellow; Y = yellow; W = white; R = red; P+ = high spectral purity; P- = low spectral purity; I+ = high intensity; I- = low intensity; UV- = UV-absorbing; UV+ = UV-reflecting).

Test no.	Stimulus 1	Stimulus 2	Test no.	Stimulus 1	Stimulus 2
1	UVB P+I+	UVB P-I+	**30**	Y P+I+	Y P-I-
2	W UV-I+	W UV+I-	**31**	UVB P-I-	UVB P+I-
3	B P+I-	B P- I-	**32**	UVY	W UV+
4	R P+I+	R P-I-	**33**	B P+I+	B P-I+
5	Y P-I+	Y P+I-	**34**	R P-I+	R P+I-
6	UVY P-I-	UVY P-I+	**35**	W UV-I-	W UV+I+
7	Y	W	**36**	UVY P+I+	UVY P+I-
8	UVB	R	**37**	R	Y
9	UVY	B	**38**	B P+I-	B P+I+
10	W UV-I+	W UV+I+	**39**	UVB P+I+	UVB P+I-
11	UVY P+I-	UVY P-I+	**40**	UVY P-I-	UVY P+I+
12	B P-I+	B P+I-	**41**	W UV-I+	W UV-I-
13	UVY	Y	**42**	Y	B
14	W UV+	UVB	**43**	UVY P+I-	UVY P-I-
15	R	B	**44**	R P+I+	R P-I+
16	Y P+I+	Y P+I-	**45**	W UV-	UVB
17	UVB P+I+	UVB P-I-	**46**	Y P-I+	Y P+I+
18	B P+I+	B P-I-	**47**	W UV-I-	W UV+I-
19	W UV-	R	**48**	UVB	UVY
20	Y	W UV+	**49**	R P+I+	R P+I-
21	UVB	B	**50**	B	W UV+
22	UVY	W UV-	**51**	Y	UVB
23	Y P-I+	Y P-I-	**52**	R	UVY
24	B P-I+	B P+I-	**53**	UVB P-I+	UVB P-I-
25	R P-I+	R P-I-	**54**	Y P+I-	Y P-I-
26	W UV+ I+	W UV+ I-	**55**	B P-I-	B P-I+
27	UVY P+I+	UVY P-I+	**56**	R P+I-	R P-I-
28	B	W UV-	**57**	W UV+I+	W UV-I+
29	R	W UV+			

CHAPTER 5

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Apis mellifera and *Tetragonula carbonaria* respond differently to colour stimuli of varied intensity and spectral purity

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Apis mellifera and *Tetragonula carbonaria* respond differently to colour stimuli of varied intensity and spectral purity

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Abstract

Bees play a vital role as pollinators worldwide and have shaped the colour of flower signals in different environments. While the Western honey bee (*Apis mellifera*, Apidae, tribe Apini) and the Buff-tailed bumble bee (*Bombus terrestris*, Apidae, tribe Bombini) are well-studied with regard to their sensory physiology and pollination capacity, little is known about stingless bees (Apidae, tribe Meliponini) from pantropical regions. Here, comparative experiments with two highly eusocial bee species, the Western honey bee, *A. mellifera*, and the Australian stingless bee, *Tetragonula carbonaria*, have been conducted to compare their colour preferences considering fine scaled perceptually similar stimuli. We made stimuli of pigment powders to allow manipulation of single colour parameters including spectral purity (saturation) or colour intensity (brightness) of a blue colour dominant wavelength (hue) for which both species have previously shown innate preferences. We studied colour preferences of free-flying honey bees (n=80) and stingless bees (n=80) and show that *A. mellifera* prefers spectrally purer colour stimuli, while colour intensity has no effect on colour choice. In contrast, however, neither spectral purity nor colour intensity affected colour preferences in *T. carbonaria* bees. These findings provide important insights into how flower community rules may be shaped by the preferences of pollinators.

Keywords: stingless bees, colour vision, spectral purity, colour intensity

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Introduction

Flower-visiting animals may develop different strategies to detect and exploit food sources. Most bees searching for food sources are limited by the distance between food source and hive, requiring efficient solutions (Visscher & Seeley 1982; Beekman & Ratnieks 2000; Greenleaf et al. 2007). Among bees, different behaviours or physiological mechanisms to locate food sources have evolved to facilitate the collection of floral rewards (Dornhaus et al. 2006; Dyer et al. 2008; Heard 2016).

Flower constancy is known for several bee species and is based on a bee's fidelity towards a specific flower type for a period of time (Free 1963; Heinrich 1979; Wells & Wells 1983; Ramalho et al. 1994; Hill et al. 1997; Slaa et al. 1998). It is hypothesized that bees increase their efficiency to collect floral rewards by visiting the same type of flowers to help ensure known reward quality or quantity (Grant 1950; Free 1963; Hill et al. 1997; Chittka et al. 1999). Nonetheless, flower constancy may have the disadvantage that workers are less flexible and may ignore more rewarding resources (Chittka et al. 1997; Dyer et al. 2014). Floral colour is assumed to have a strong impact on flower constancy in honey bees (Hill 1997; Banschbach 1994; Gegear & Laverty 2004).

Honey bees are able to make very fine colour discriminations (von Helversen 1972; Dyer & Neumeyer 2005; Papiorek et al. 2013), but have coarse spatial acuity for colour stimuli (Giurfa et al. 1996), while bumble bees have more coarse discrimination but have a higher visual acuity to find rewarding flower resources (Dyer et al. 2008; Morawetz & Spaethe 2012; Bukovac et al. 2017a). Probably, the trade-off between colour discrimination and colour detection evolved based on the respective foraging behaviour. Bumble bees forage on widely dispersed flower patches, while honey bees usually visit mass-flowering resources (Dornhaus &

Chittka 1999, 2004; Heinrich 2004). The higher visual acuity of bumblebees helps with the detection of small or sparse resources and is less advantageous in habitats with abundant resources. Furthermore, the waggle dance of honey bees provides no gain in patchy habitats, where individual target detection is more advantageous (Dornhaus & Chittka 1999, 2004; Sherman & Visscher 2002). Thus it is plausible that bees from different environmental conditions have different ways of processing colour signals. Colour signals are complex and colour may be described by several key parameters including hue, saturation and brightness (Kemp et al. 2015).

Many studies have analysed colour preferences in honeybees and have concluded that colour stimuli with a "blue" hue (UV-blue, blue, blue-green) are preferred by honey bees and that the blue contrast interferes with shape learning (Menzel 1967; Giurfa et al. 1995; Morawetz et al. 2013). In a series of experiments, Rohde et al. (2013) trained honey bees and bumble bees to a certain colour stimulus and offered two additional stimuli, one with higher and another with lower spectral purity, in a subsequent test. Both bee species chose the higher degree of spectral purity significantly more often than the trained colour stimulus. These findings correspond to earlier results with bumble bees and lead to the assumption that spectral purity is an important parameter for colour choice in bumble bees and honey bees (Lunau 1990), and potentially bee colour perception in general. If this finding is consistent for other bee species it may explain how bees find and choose flowers in a way that could explain flower community assemble (Kantsa et al. 2017).

The influence of colour preferences on how stingless bees may choose flowers is still unclear. Brazilian stingless bees of the genus *Melipona* showed preferences for specific colour hues (yellow and UV-blue) while choices were not significantly influenced by the intensity or spectral purity of stimuli (Koethe et al. 2016). In an experiment by Dyer et al. (2016a) innate preferences of the Australian stingless bee *Tetragonula carbonaria* were analysed by employing broadband colour stimuli from different regions of colour space, and like in honeybees, stimuli from the blue and blue-green regions of colour space were preferred. Furthermore, a combination of green contrast and spectral purity seemed to influence the workers preferences (Dyer et al. 2016a), although spectral purity as a single factor was not a significant factor explaining the observed behaviour. In general, intensity is assumed to play a minor role in colour choice by bees (Daumer 1956; Backhaus 1991; Spaethe et al. 2001; Reser et al. 2012; Ng et al. 2018; Van der Kooi et al. 2018) although intensity has been considered a potentially important factor for flower evolution (Hopkins & Rausher 2012; Renoult et al. 2014; Sletvold et al. 2016) and an experimental approach by Hempel de Ibarra et al. (2000) did show that the very high brightness contrast between a stimulus and its background can impact the choice behaviour of bees. Achromatic perception of targets is driven by green contrast (modulation of green receptor against the background) in bees and is considered to play an important role in shape processing and motion perception (von Hess 1913; Kaiser & Liske 1974; Lehrer & Bischof 1995; Hempel de Ibarra & Giurfa 2003; Stach et al. 2004; Stojcev et al. 2011; Morawetz et al. 2013). Furthermore, in several studies green contrast influenced choice behaviour of both honey bees and stingless bees (Giurfa et al. 1996, 1997; Dyer et al. 2016a).

In the past two decades, research of stingless bees and native bee species has received increased interest as crop pollinators due to the decline of honey bee colonies (Heard 1999; Amano et al. 2000; Slaa et al. 2000; Kremen et al. 2002, 2004; Slaa et al. 2006, Nunes-Silva et al. 2013; Barbosa et al. 2015). Stingless bees are known to pollinate several crops and in some cases the pollination service by stingless bees is more efficient than by honey bees (Cruz et al. 2005; Dos Santos et al. 2009). Nonetheless, whilst honey bees have been researched for over 100 years (Dyer & Arikawa 2014), the available data on stingless bees are still rather sparse.

Previous studies concerning the visual capabilities of stingless bees mostly investigated colour choice behaviour with regard to known preferences of model organisms like *A. mellifera* and *B. terrestris* (Dyer et al. 2016a; Koethe et al. 2016). The colour discrimination of temperate species (*A. mellifera* and *B. terrestris*) is finer than in pantropical bee species (Meliponini), although analyses of flower spectral signals in temperate and pantropical regions are almost identical (Chittka & Menzel 1992; Arnold et al. 2010; Dyer et al. 2012; Shrestha et al. 2013, 2014; Bukovac et al. 2017a). So far, studies analysing colour vision in bees suggest that temperate and pantropical bee species may share preferences for blue colour hues, and maybe also the spectral purity of colours, as honest indicator of flowers offering nectar rewards (Menzel 1967; Chittka & Menzel 1992; Kantsa et al. 2017; Koethe et al. 2016). Currently however, it is unknown the extent to which such preferences are common for bees around the world in a way that could be a major driver of flower colour.

Here, we analysed whether the Australian stingless bee (*Tetragonula carbonaria* Smith) and the Western honey bee (*A. mellifera* Linnaeus) choose colours according to the colour parameters spectral purity (saturation) or intensity (brightness) by employing stimuli sets that had the same hue but differed either in their spectral purity or intensity. It is currently unknown to what extent these colour parameters might act as drivers for the evolution of flower colours, but Australian flowers have spectral signatures that are nearly identical to the Northern Hemisphere where the Western honeybee has long been an influential pollinator, and so the comparison of preferences in key bee species provides significant insights into the process of flower signalling [68]. Spectral purity varies due to pigment concentration and bumblebees as well as honeybees are known to prefer pigment concentrations that yield the highest degree of spectral purity, but the conspicuousness of flowers might be more dependent on colour intensity in forest as compared to open habitats (Papiorek et al. 2013; Binkenstein & Schaefer 2015). Alternatively, detection via colour signaling may be more important than honest signaling of reward, and so it is important to understand the extent to which bees in respective environments demonstrate colour preferences for spectrally pure signals (Bukovac et al. 2017a).

Material and Methods

Manufacture of colour stimuli

The manufacture of stimuli to manipulate single colour parameters was enabled using artist pigments (introduced by Koethe et al. 2016, 2018). Two blue artist pigments were blended to determine the hue of the stimuli (Artist Pigments: “Sky Blue”, “Ultramarine Blue”, Art Material International Warenhandelsgesellschaft mbH, Kaltenkirchen, Germany). For the manipulation of colour intensity and spectral purity, barium sulphate (white), black pigment, or a mixture of both achromatic powders, were added to the blue blend (barium sulphate 99 % pure, Grüssing GmbH Analytika, Filsum, Germany; “DeiArt Russverkollerung”, Deifel GmbH & Co. KG, Schweinfurt, Germany). The powders were compacted into culture dishes (35 mm in diameter, 10 mm in height) using a custom-build pigment press. Various combinations of colour intensity and spectral purity were fabricated by mixing defined amounts of the blue blend and varying amounts of white, grey and/or black powders. The resulting spectral reflectances were measured via spectrometer analysis (USB4000 miniature fibre optic spectrometer, Ocean Optics GmbH, Ostfildern, Germany) at an angle of 45° using a UV-NIR deuterium halogen lamp (DH-2000-BAL, Ocean Optics GmbH), which was connected to the spectrometer by a UV-VIS fibre optic cable (Ø 600 µm, QR600-7-UV 125 BX, Ocean Optics GmbH). To calibrate the spectrometer a black standard (black PTFE powder, Spectralon diffuse reflectance standard SRS-02-010, reflectance factor of 2.00 %, Labsphere, Inc. North Sutton, USA) and a white standard (white PTFE powder, Spectralon diffuse reflectance standard SRS99-010, reflectance factor of 99.00 %, Labsphere, Inc. North Sutton, USA) were used (Fig. 1). The obtained spectral data were plotted in the colour hexagon model by Chittka (1992) and the hue (dominant wavelength) and the spectral purity of the colours were calculated from the perceptual distance between a colour locus and the background in relation to the distance between the background and the spectral line $SP = \frac{H_i(target-background)}{H_i(spectral\ locus-background)}$ (Lunau et al. 1996) (Fig. 1). The intensity was calculated by adding up the receptor excitation values for all three photoreceptors and dividing those by the number of photoreceptors [$I = (E_{UV} + E_B + E_G)/3$]. Green contrast was given by the photoreceptor excitation of the green receptor minus 0.5 [$GC = E_G - 0.5$] (Spaethe et al. 2001). Based on the results of these calculations four stimuli with differing levels of spectral purity but the same intensity level, and *vice versa*, were selected (Online Resource 1). The hue of all stimuli was the same. Each stimulus was covered by an UV-transmitting Plexiglas discs with an indentation in the centre to offer 10 µl sugar solution (30-50%).

Experimental arena

To test the bees in a controlled surrounding, an arena was constructed by using grey UV-reflecting wallpaper (for reflectance curve see: Fig. 1; Climapor Insulation Wallpaper Graphite, Saarpor, Neunkirchen, Germany). A 50 x 50 cm plywood board was covered with the same wallpaper and a 13.5 cm high circular wall consisting of the same wallpaper with a diameter of 50 cm was constructed.

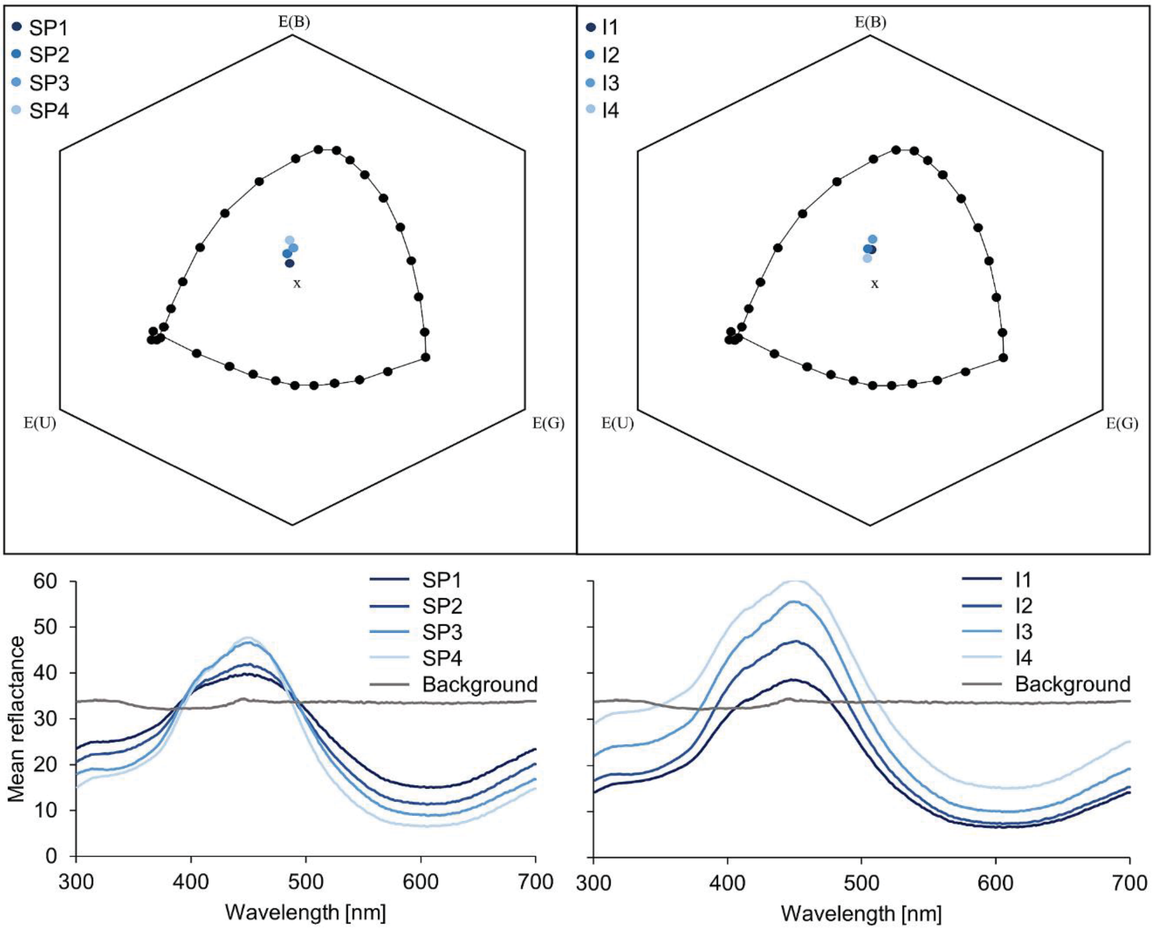


Fig 1. Colour hexagon and reflectance curves

Upper row: The colour hexagon according to Chittka (1992) displays the perception of colours in accordance with bee-specific photoreceptor sensitivities (*Apis mellifera*), the background (grey Styrofoam wallpaper) and the ambient light (standard daylight illumination D65). Lower row: Reflectance curves of all colour stimuli (left: stimuli with manipulated spectral purity; right: stimuli with manipulated colour intensity; background = UV-reflecting grey wallpaper).

By choosing this height of the wall colour perception was enabled and the sole perception by green contrast was disabled (Online Resource 2).

The stimuli were positioned randomly by dividing the arena in six columns and six rows resulting in 36 positions. By rolling a dice the coordinates for each stimulus during training and test were randomly determined; repeated identical positions were re-randomised. The distance between the stimuli was 3 cm. To avoid an influence of casted shadows or proximity to the wall the outer edges of the arena were not taken into consideration for placement of stimuli and the position of the arena was adjusted to avoid shadow casting.

Conditioning phase – *A. mellifera*

Honey bees were recruited from university maintained hives located at the Botanical Garden of the Heinrich-Heine University Düsseldorf, Germany. The bees were freely flying and hence flower experienced. Workers of *A. mellifera* were trained to a feeder outside the arena because their recruitment was very effective and would have led to many workers inside the arena at the same time. As the number of bees varied strongly the concentration of sugar solution was adjusted to attract more (30%) or fewer bees (10%). After workers returned frequently to the feeder, the stool on which the feeder was positioned was relocated approximately 5m towards the area where the training and test took place. The area to which the bees were directed was a shady meadow under some trees with constant light conditions. Single workers were trained to the arena by using higher concentrated sugar solution (30-50%, depending on the sugar concentration in the feeder). Each trained bee was marked individually with nail polish on its dorsal abdomen. For the training four identical stimuli were used (SP1 or SP4; I1 or I4) thus promoting absolute conditioning to participants in the experiment (Dyer & Chittka 2004; Giurfa 2004). After each visit to a stimulus the Plexiglas disc was replaced by a clean disc. Each training of *A. mellifera* consisted of three to four foraging bouts and in each foraging bout the workers could visit up to four training stimuli (8-13 visits per bee per training). Each individual worker was either trained to the lowest ranked stimulus (n = 20) of intensity (I1) /spectral purity (SP1) or to the highest ranked stimulus (n = 20) of the referring parameter (I4/SP4) (Online Resource 3 B-C). Each worker had to participate in two trainings and two tests. After a worker was trained to the lowest ranked stimulus of a parameter (I1 or SP1) and tested successfully it was retrained to the highest ranked stimulus of the respective parameter (I4 or SP4) and *vice versa*. Thus, 20 individuals were trained first to the lowest ranked stimuli (purity or intensity), and 20 individuals were first trained to the highest ranked stimuli, so that in total 80 honey bees were trained and tested. If more than one bee returned to the arena all additional bees were captured in tubes and released after finishing the experiment with the first bee. After completing the experiment each worker was sacrificed to avoid pseudo-replication of data.

Conditioning phase – *T. carbonaria*

Hives of *T. carbonaria* – provided by Sugarbag Bees (sugarbag.net) – were kept in an urban environment of Brisbane, Australia. A gravity feeder made of UV-transmitting Plexiglas was placed in the middle of the arena to attract workers of *T. carbonaria* (Online Resource 3 A). If more than one bee returned to the gravity feeder all additional bees were captured in tubes and released after finishing the experiment with the first bee. The released workers returned willingly to the arena and could be trained and tested. Workers of T. *carbonaria* are much smaller than workers of *A. mellifera* and therefore visited only one stimulus per foraging bout. Each worker of *T. carbonaria* was trained for eight foraging bouts to ensure a comparable training effect compared to honey bees. As with honey bees, four identical stimuli were used for training. Two training approaches per parameter were also used for *T. carbonaria* with either the lowest ranked stimulus (I1 or SP1) or the highest ranked stimulus (I4 or SP4) in the beginning, like in honeybees. In total, 80 workers of *T. carbonaria* were tested (first training I1 (n = 20); first training I4 (n = 20); first training SP1 (n = 20); first training SP4 (n = 20)).

After completing the second test each worker was sacrificed to avoid pseudo-replication.

Test phase

Each bee had to complete two test rounds. A total of 40 workers of each species were tested regarding intensity – based on trainings starting with high ranked stimuli (n =20) and trainings starting with low ranked stimuli (n = 20) – and another 40 workers of each species were tested regarding spectral purity – based on trainings starting with high ranked stimuli (n = 20) and trainings starting with low ranked stimuli (n = 20) –, resulting in 80 workers per species in total. The second test was based on a reciprocal training. In each test eight stimuli were presented (two stimuli per level) that offered water instead of a sugar solution reward and five decisions per bee of this unrewarding test were recorded (Online Resource 3 D).

Statistical analysis

The statistical program R was used to analyse the data (R Development Core Team 2008). All data were tested for normal distribution by using the Shapiro-Wilk test.

In order to test whether the choices of the first and second test that were based on training with the same stimulus can be pooled Z-transformation according to Fisher was used. Therefore, the choices of a test were correlated with the levels of the respective colour parameter (intensity or spectral purity) using Pearson's correlation. Based on the Pearson's correlation coefficient (*r*) the z and σ (standard error) values were calculated:

$$z = 0.5 * (\ln(1 + r) - (\ln(1 - r))$$

$$\sigma = \frac{1}{\sqrt{n-3}}$$

Based on the z and σ values the lower and upper confidence limits (95% confidence interval) were calculated as followed:

$$z - 1.96 * \sigma$$

$$z + 1.96 * \sigma$$

The 95% confidence intervals were used to establish if data from the same preliminary training overlapped and were thus not significantly different, which enabled pooling of data from the subsequent tests.

The data were collected for 80 bee individuals of *A. mellifera* and 80 individuals of *T. carbonaria*. The data were analysed by testing the bees' consecutive choices (the first five choices per bee) for the different stimuli of each parameter (intensity and spectral purity) using a generalised linear mixed model (GLMM). We used the "lme4" package of R to analyse the individual choices of the bees, which were assessed using GLMM with Poisson distribution of data and the best linear fit depending on Akaike Information Criterion (AIC) score (R Development Core Team 2008, Bates et al. 2009). We analysed the number of choices for each stimulus as fixed effect and the individual bees were used as random effect of the model. To test the distribution of choices between the four stimuli of each stage a multiple comparison of means was done with Tukey all-pair comparisons.

Results

The analysis using Z-transformation according to Fisher resulted in different colour choices depending on whether workers were conditioned to a stimulus in the first or second training for *T. carbonaria,* but not for *A. mellifera* (Online Resource 4). Therefore, pooling the data of *T. carbonaria* is inadequate and all data were analysed based on the preliminary training.

When workers of *T. carbonaria* were initially trained on the stimulus with highest spectral purity (SP4) they chose SP4 more often than SP1 in the subsequent test (Fig. 2; first test (SP4): GLMM: n = 20; p = 0.010). In the second test based on training to SP4 the workers chose randomly (second test (SP4): GLMM: n = 20; p = 0.920). When trained on the least spectrally pure stimulus (SP1) the workers show no preference for a specific stimulus (first test (SP1): GLMM: n = 20; p = 0.363; second test (SP1): GLMM: n = 20; p = 0.867).

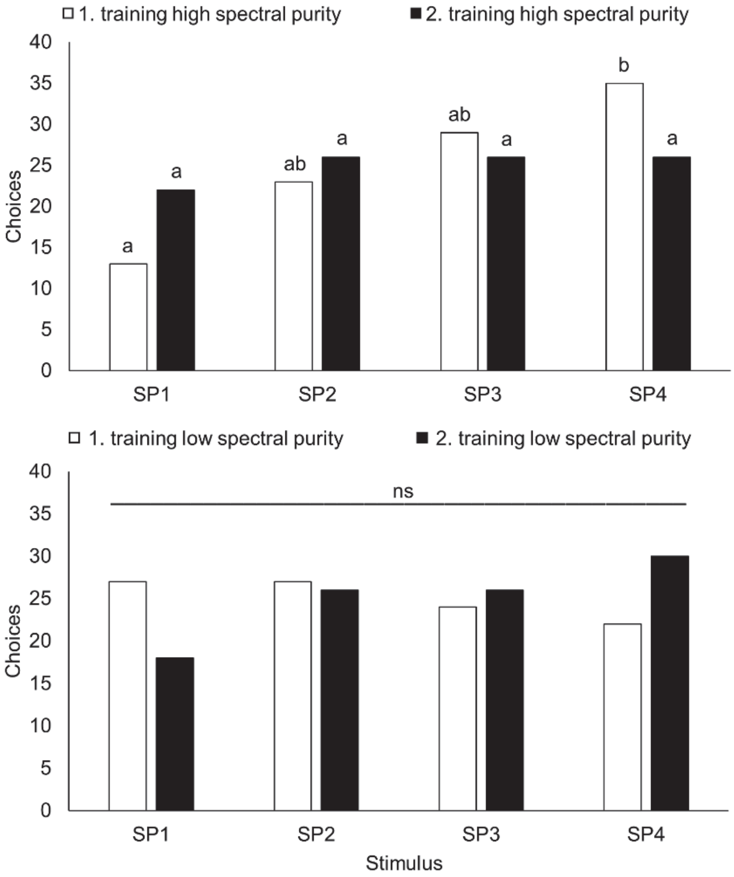


Fig 2. Colour choices with manipulated spectral purity of *T. carbonaria*
Top: Total choices made by workers of *T. carbonaria* after training to the stimulus with highest spectral purity (SP4) in the first (white, n=20) or second (black, n=20) training. Bottom: Total choices made by workers of *T. carbonaria* after training to the stimulus with lowest spectral purity (SP1) in the first (white, n=20) or second (black, n=20) training. Different letters above the columns indicate significant differences (p<0.05; ns = not significant; Tukey all-comparison test).

The choices of *T. carbonaria* concerning the stimuli with varying intensity resulted in no depicted preference for any of the four stimuli (Fig. 3; first test (I4): GLMM: n = 20; p = 0.112; second test (I4): GLMM: n = 20; p = 0.275; second test (I1): n = 20; p = 0.348). The workers that were first trained to I1 chose the stimulus with the second lowest intensity (I2) most often and significantly more often than I4 (first test (I1): GLMM: n = 20; p = 0.013).

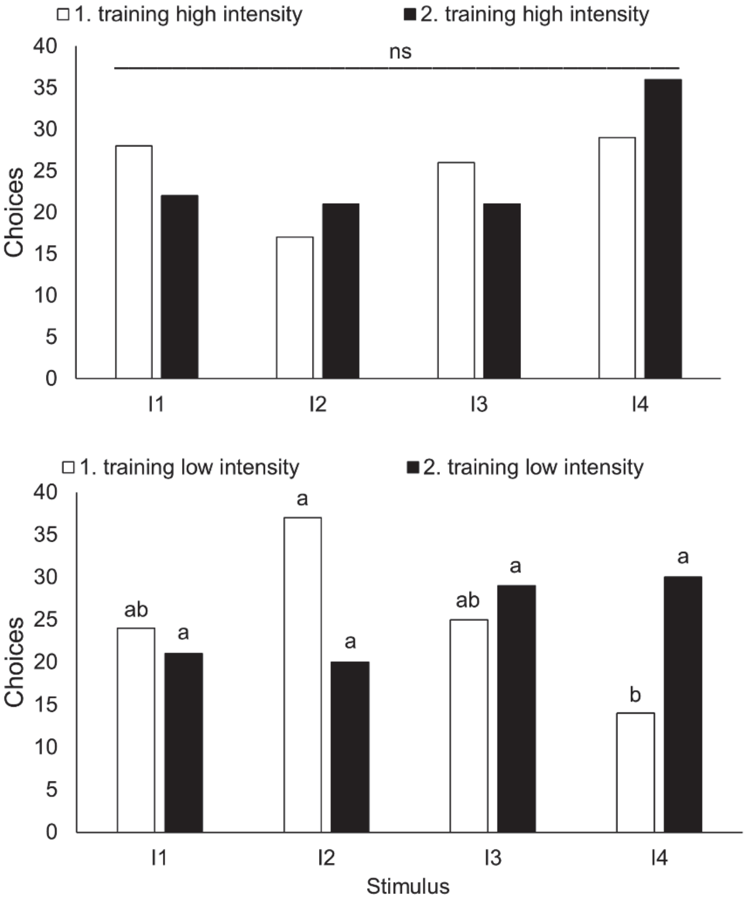


Fig 3. Colour choices with manipulated intensity of *T. carbonaria*
Total choices made by workers of *T. carbonaria* after training to the stimulus with highest intensity (I4, n=20) in the first (white, n=20) or second (black, n=20) training. Bottom: Total choices made by workers of *T. carbonaria* after training to the stimulus with lowest intensity (I1) in the first (white, n=20) or second (black, n=20) training. Different letters above the columns indicate significant differences (p<0.05; ns = not significant; Tukey all-comparison test).

In comparison, workers of *A. mellifera* preferred stimuli with high spectral purity (Fig. 4). In the first test, based on the previous training to SP4, the workers of *A. mellifera* chose the stimuli according to spectral purity. The higher the value of spectral purity the more often the stimuli were chosen (first test (SP4): GLMM: n = 20; p < 0.001). In the second test, the bees chose similar to the first test (second test (SP4): GLMM: n = 20; p < 0.001). When the bees were initially trained to the least spectrally pure stimulus the preference for spectrally purer colours was reduced, but there still remained a significant difference that honeybees chose the stimulus of lowest spectral purity least in the second test based on training to SP1 (first test (SP1): GLMM: n = 20; p = 0.059; second test (SP1): GLMM: n = 20; p < 0.001).

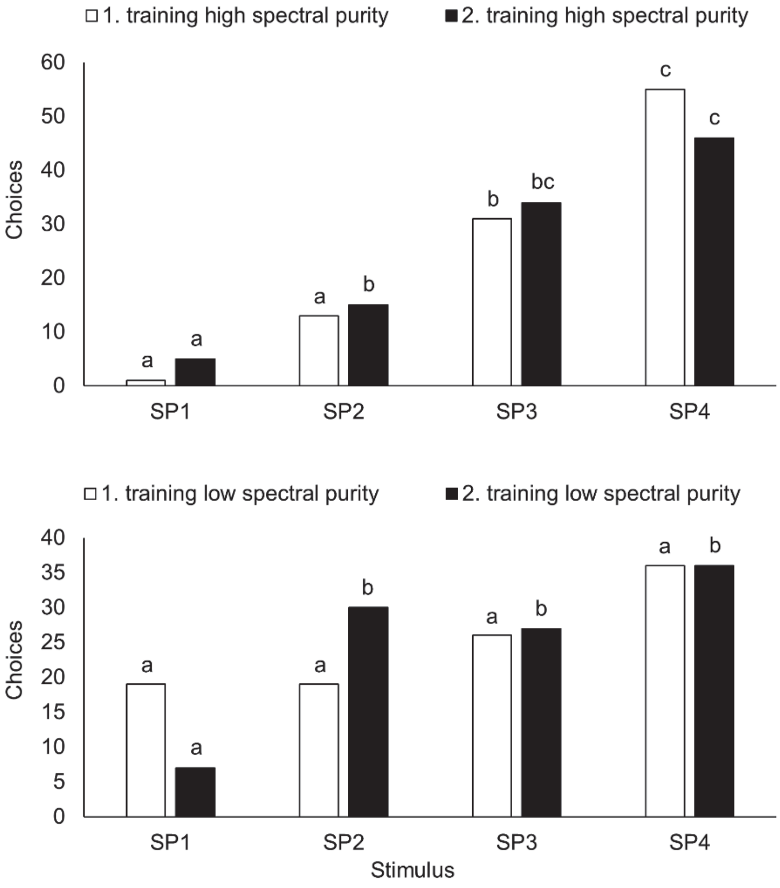
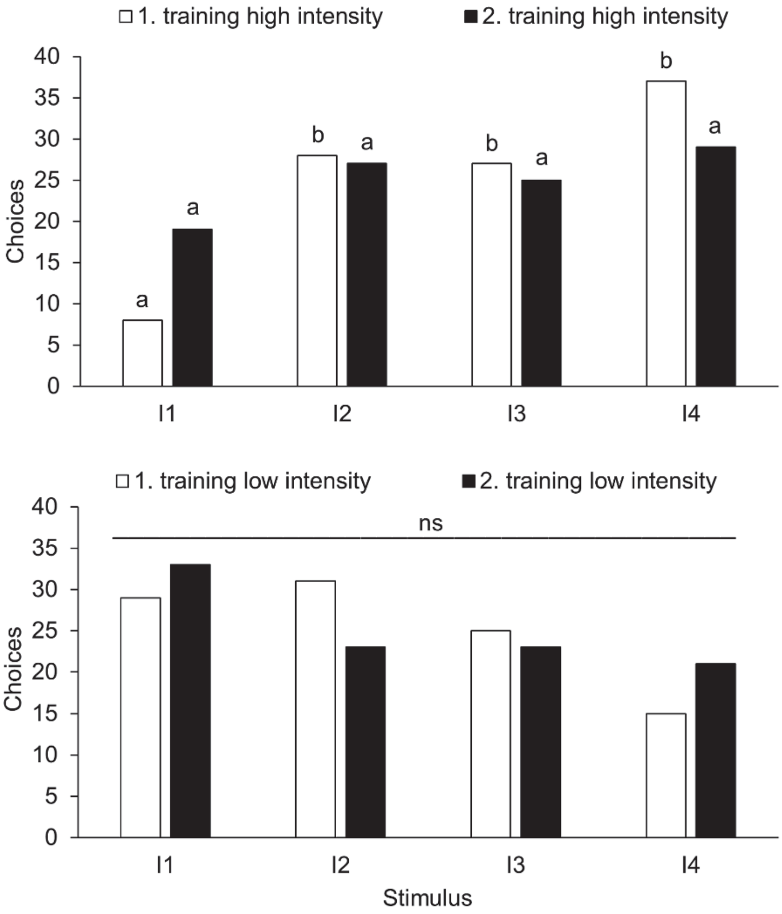


Fig 4. Colour choices with manipulated spectral purity of *A. mellifera*

Top: Total choices made by workers of *A. mellifera* after training to the stimulus with highest spectral purity (SP4) in the first (white, n=20) or second (black, n=20) training. Bottom: Total choices made by workers of *A. mellifera* after training to the stimulus with lowest spectral purity (SP1) in the first (white, n=20) or second (black, n=20) training. Different letters above the columns indicate significant differences (p<0.05; ns = not significant; Tukey all-comparison test).

Concerning the stimuli with varying intensities, the workers of *A. mellifera* preferred more intense stimuli when initially trained to I4 over I1, while their choices were random in the second test and in both tests following training to I1 (Fig. 5; first test (I4): GLMM: n = 20; p < 0.001; second test (I4): GLMM: n = 20; p = 0.506; first test (I1): GLMM: n = 20; p = 0.085; second test (I1): GLMM: n = 20; p = 0.344).

Fig 5. Colour choices with manipulated intensity of *A. mellifera* Total choices made by workers of *A. mellifera* after training to the stimulus with highest intensity (I4) in the first (white, n=20) or second (black, n=20) training. Bottom: Percentage choices made by workers of *A. mellifera* after training to the stimulus with lowest intensity (I1) in the first (white, n=20) or second (black, n=20) training. Different letters above the columns indicate significant differences (p<0.05; ns = not significant; Tukey all-comparison test).



Discussion

The comparison of colour preferences between honey bees and stingless bees suggests that there are distinct differences in how the respective bee species process the saturation of colour stimuli when choosing stimuli. The results of honey bees are consistent with previous results that honey bees prefer spectrally purer colours (Papiorek et al. 2013; Rohde et al. 2013). In contrast, the stingless bees preferred none of the four stimuli of neither colour intensity nor spectral purity. This result for stingless bees is consistent with previous testing with dissimilar colours that also reported that spectral purity as a single factor did not explain bee choices (Dyer et al. 2016a), although in that experiment the resolution to detect potentially small changes in colour preferences was limited. In the current study considering the preferred colour hue of stingless bees, colour choices were independent from manipulated colour parameters of either spectral purity or intensity.

In honey bees, variations in colour intensity did not significantly influence preferences. In a recent study, Ng et al. (2018) tested the choice behaviour of honey bees, in which the bees were challenged to detect bee-achromatic stimuli based on intensity when presented at a visual angle to promote colour processing. The bees failed to detect the stimuli, although they were very accurate at detecting control stimuli containing chromatic contrast. These findings that honeybees do not process stimulus intensity for either colour preference or colour detection tasks agrees with previous work that stimulus intensity is not processed in colour discrimination tasks by free flying honeybees (Daumer 1956; Backhaus 1991; Reser et al. 2012).

One reason for the disparity in colour choice between honey bees and stingless bees could be that the values of both colour parameters were calculated according to the photoreceptor sensitivities of honey bees and perhaps the stingless bees were less able to distinguish the used colours. But since photoreceptor sensitivities in bees are highly conserved this possibility seems unlikely (Peitsch et al. 1992; Chittka 1996; Briscoe & Chittka 2001; Spaethe et al. 2014; Dyer et al. 2016b). The photoreceptor sensitivities of *T. carbonaria* have not been analysed yet. The reflectance of the used stimuli was reanalysed by using the available photoreceptor sensitivities of *Trigona spinipes*, another species of stingless bees (Meliponini) as suggested by previous studies on colour perception in stingless bees (Spaethe et al. 2014; Dyer et al. 2016b), to get more appropriate values for *T. carbonaria*. The values calculated for *A. mellifera* and *T. spinipes* are nearly identical (Online Resource 1) and therefore, the values are assumed to be reliable for *T. carbonaria*.

Honey bees and stingless bees use elaborate communication to inform nestmates about the whereabouts of profitable food sources. The dance language of honey bees is well-studied and highly efficient (Dyer 2002; von Frisch 1967). Foragers can be recruited to high quality food sources, but honey bees are also known to forage individually Seeley 1986, 1994). Many stingless bee species including stingless bees are known to forage in groups to exploit food sources Nieh 1999; Jarau et al. 2003; Biesmeijer & Slaa 2004; Flaig et al. 2016). This foraging behaviour – known as mass recruitment – could explain the absent preference for small changes in colour parameters like offered in this experiment. Stingless bees in general use elaborate chemical communication that could influence the choice of workers more strongly than colour information but they also rely on other communication strategies, e.g. piloting or excited movements Nieh 1999; Nieh & Roubik 1998; Jarau 2009). Single bees that search for new food sources – so called scout bees – could be influenced by floral colours but in the following recruitment process these information could play a minor role (Hubbell & Johnson 1978). Both investigated species in the current study are generalist flower visitors but the more scent driven communication of stingless bees could induce a less visually driven choice while honey bees are able to communicate more detailed information about food sources via their dance language (Dyer 2002). Indeed, recent work on flower communities suggests that flower colour and olfactory signals are often coordinated to help bees find flowers (Kantsa et al. 2017, 2018), and detecting a flower colour signal may be more important than fine-grained colour processing of spectral content (Bukovac 2017a). The conducted experiments minimize the effect of scent driven decisions because the stimuli were cleaned after each landing and their positions were randomly changed, thus providing access to the visual factors mediating flower preferences.

A study by Streinzer et al. (2016) showed that the body size of stingless bees correlates with their ability to forage in dim-light conditions. Stingless bees with smaller bodies and hence smaller eyes and lower numbers of ommatidia are less sensitive to low light conditions than larger species. In general, stingless bee species are smaller than honey bees but larger stingless bee species reach the size of honey bee workers (Wille 1983; Jarau & Barth 2008). *T. carbonaria* is much smaller than *A. mellifera* (worker body length of 4 mm and 15 mm, respectively) and therefore it could be possible that the visual capacities of *A. mellifera* are better (Online Resource 3 E-F). Furthermore, stingless bees have a reduced flight range compared to honey bees and a reduced foraging area – hence reduced number of food sources in the area – could make specific preferences unnecessary (Beekman & Ratniek 2000; Greenleaf et al. 2007; van Nieuwstadt & Iraheta 1996; Araújo et al. 2004). Most small stingless bees do not only forage fewer hours per day but also forage in a smaller area since their flight range is relatively small.

In previous studies, stingless bees showed weak but significant preferences for blueish colour hues considering stimuli with high colour contrasts (Dyer et al. 2016a; Koethe et al. 2016). Only *Partamona helleri* showed a distinct preference for spectrally purer colours when choosing stimuli with the same colour hue (Koethe et al. 2018). In a study conducted in a Mediterranean scrubland, the amount of nectar and the degree of spectral purity positively correlated and hence provide a possible explanation for a preference of more spectrally pure colours in flower visitors like found in honey bees (Kantsa et al. 2017), thus suggesting there may be regional differences in how different bee preferences may affect local communities.

It has been previously discussed whether innate colour preferences or learned behaviour have a bigger impact on the colour choice of bees for flowers (Menzel 1963; Heinrich et al. 1977; Hill et al. 1997; Gumbert 2000). In the current experiments, the results for either *A. mellifera* or *T. carbonaria* are only slightly influenced by the training but remain consistent. While *T. carbonaria* did show some significant choices driven by training but not based on depicted colour preferences, *A. mellifera* significantly preferred stimuli of high spectral purity less dependent on the previous training. It seems possible that *T. carbonaria* has less emphasized colour preferences than *A. mellifera* or is more strongly influenced by experience. The test results of the first and second test based on the same training stimulus could be pooled for *A. mellifera* in all cases, while for *T. carbonaria* the results differed based on whether it was the first or second test. Additionally, when comparing the first choices made by each worker with the total choices (five per worker) the results for both species are consistent (Online Resource 5 & 6).

T. carbonaria inhabits open forest and woodland areas, while also commonly sharing habitats with humans (Heard 2016). The light conditions in these areas are very diverse and could have influenced colour perception. It is hypothesized that bees' visual perception is adapted to their respective environment (Bukovac et al. 2013). If so, bee species like *T. carbonaria*, that live in areas with unsteady light conditions could show broader colour choices and could be less influenced by small changes in colours than bees that are more adopted to steady light conditions. *A. mellifera* is considered a tropical and temperate bee since they can be found on every continent, except Antarctica, in various habitats and genetic analysis had shown adaptations of honey bee populations according to their habitat (Sherman & Visscher 2002; Dornhaus & Chittka 2004; Whitfield et al. 2006). These adaptations may lead to a more or less distinct colour differentiation between fine scaled colour differences as used here, and thereby promote distinct colour preferences or generalization of fine scaled colour differences.

A recent study by Garcia et al. (2017) compared colour processing of representatives of three tribes of bees including honey bees (*A. mellifera*, tribe Apini), bumble bees (*B. terrestris*, tribe Bombini) and stingless bees (*T. carbonaria* and *Trigona cf. fuscipennis*, tribe Meliponini) and showed that although the photoreceptor sensitivities are highly preserved, the processing of colour differs among different bee species. Interestingly, however, even in honeybees that have relatively fine colour discrimination compared to stingless bees, there is some plasticity in colour discrimination depending upon conditioning that appears to cater for the natural variability in flower colour pigments (Garcia et al. 2017, 2018).

Colour signals of angiosperms are similar in temperate and pantropical regions of the world and the colour perception of bees and other pollinators has a major impact on the evolution of floral colours (Bukovac et al. 2017a; Dyer et al. 2012; Shrestha 2013). Nonetheless, colour preferences of temperate and pantropical bee species appear to be quiet divergent. While temperate bees prefer spectrally purer colours, pantropical bees ignore spectral purity in many cases as a decisive factor (Papiorek et al. 2013; Rohde et al. 2013; Dyer et al. 2016a; Koethe et al. 2016, 2018). Several studies – including this one – dismissed colour intensity as a key parameter for colour preferences of bees (Daumer 1956; Backhaus 1991; Reser et al. 2012; Koethe et al. 2018; Ng et al. 2018). Spectral purity was assumed to be a key parameter for temperate bee species, but cannot be regarded a key parameter for pantropical bee species (Papiorek et al. 2013; Rohde et al. 2013; Dyer et al. 2016a; Koethe et al. 2016, 2018). Pantropical bees may not rely on single colour parameters or the preference for spectrally purer colours in temperate bee species is an acquired side effect of other factors, e.g. higher nectar amount (Kantsa et al. 2017). In comparison, the results suggest that colour preference for spectrally pure colour signals are not the major driver of flower colour evolution at a global level, which fits with some recent evidence that flower spectra are clustered in certain regions of the spectrum or colour space so as to best promote initial detection, and then subsequent discrimination of flowers (Bukovac et al. 2017a; 2017b). Indeed, recent studies on flower community assembly rules in a Mediterranean scrubland in Greece show flowers with particular colours frequently emit similar scents and the resulting facilitation for pollinators to find flowers appears the main driver of the evolutionary process, rather than direct competition between plant species (Kantsa et al. 2017, 2018).

In conclusion, colour preferences among the two tested bee species are disparate although their photoreceptor sensitivities are highly conserved (Peitsch et al. 1992; Chittka 1996; Briscoe & Chittka 2001). Therefore, the processing of colours, the environmental challenges of a habitat and behavioural influences (e.g. communication, foraging strategies etc.) may impact colour choices of different bee species in a variety of ways.

Competing interest

We have no competing interest.

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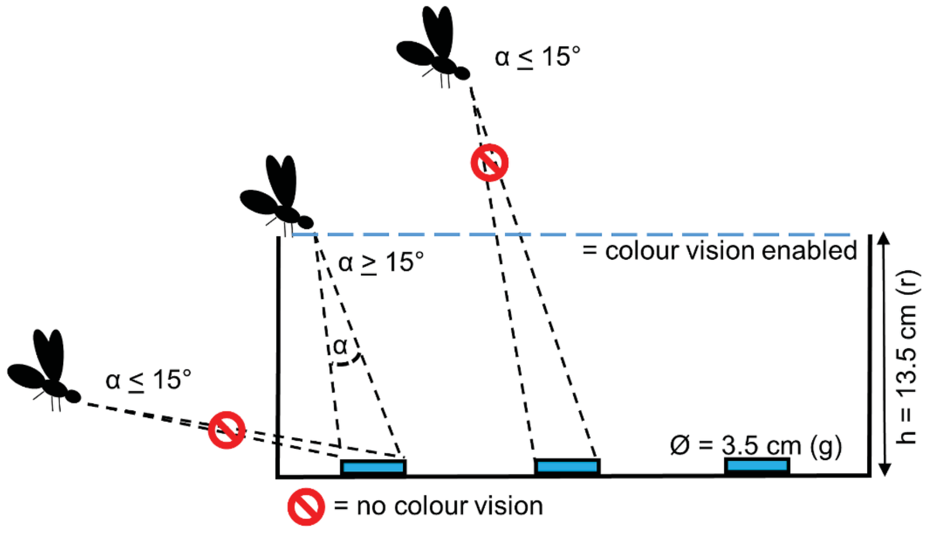
Supplement

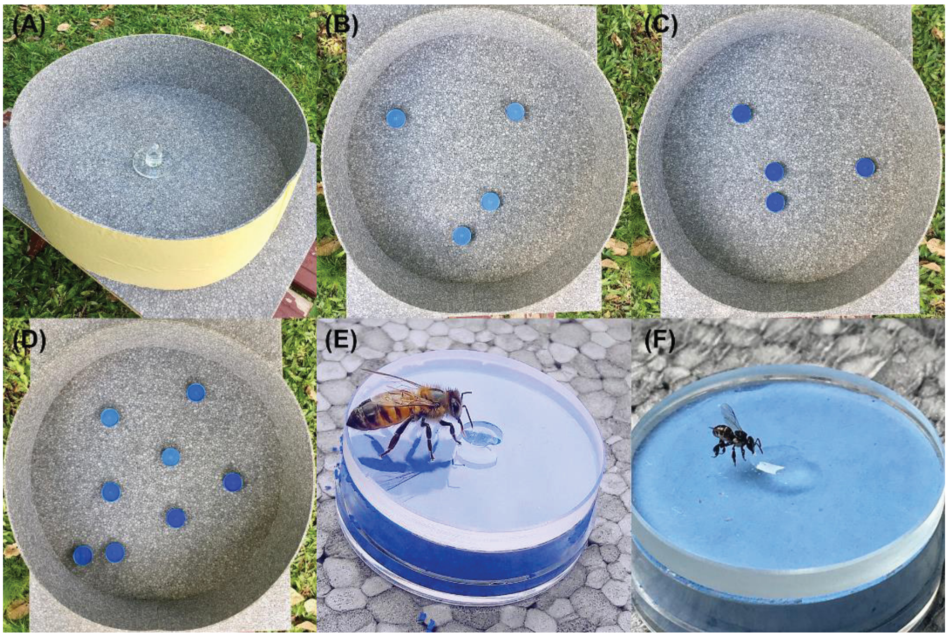
Online Resource 1 Calculated colour parameter based on photoreceptor sensitivities of *Apis mellifera* and *Trigona spinipes*

Apis mellifera	**I1**	**I2**	**I3**	**I4**	**SP1**	**SP2**	**SP3**	**SP4**
UV receptor	0.3791	0.4104	0.4683	0.5219	0.4581	0.4412	0.4182	0.4037
Blue receptor	0.4983	0.5440	0.5852	0.6127	0.5235	0.5293	0.5451	0.5433
Greenreceptor	0.3617	0.3992	0.4467	0.4954	0.4421	0.4226	0.4156	0.3890
Colour contrast	0.1288	0.1395	0.1291	0.1065	0.0748	0.0987	0.1282	0.1475
Green contrast	-0.1383	-0.1008	-0.0533	-0.0046	-0.0579	-0.0774	-0.0844	-0.1110
Blue contrast	-0.0017	0.0440	0.0852	0.1127	0.0235	0.0293	0.0451	0.0433
Brightness contrast	1.2391	1.3536	1.5002	1.6300	1.4236	1.3931	1.3789	1.3360
Spectral purity	0.2734	0.2955	0.2857	0.2459	0.1694	0.2177	0.2721	0.3154
Intensity	0.4130	0.4512	0.5001	0.5433	0.4745	0.4644	0.4596	0.4453
Trigona spinipes	**I1**	**I2**	**I3**	**I4**	**SP1**	**SP2**	**SP3**	**SP4**
UV receptor	0.3750	0.4046	0.4643	0.5199	0.4569	0.4389	0.4400	0.3979
Blue receptor	0.4962	0.5416	0.5833	0.6109	0.5224	0.5278	0.5794	0.5409
Green receptor	0.3416	0.3774	0.4255	0.4776	0.4300	0.4068	0.4311	0.3658
Colour contrast	0.1410	0.1524	0.1424	0.1180	0.0823	0.1086	0.1440	0.1614
Green contrast	-0.1584	-0.1226	-0.0745	-0.0224	-0.0700	-0.0932	-0.0689	-0.1342
Blue contrast	-0.0038	0.0416	0.0833	0.1109	0.0224	0.0278	0.0794	0.0409
Brightness contrast	1.2128	1.3235	1.4731	1.6084	1.4093	1.3735	1.4506	1.3046
Spectral purity	0.2807	0.2966	0.2946	0.2545	0.1731	0.2308	0.2807	0.3333
Intensity	0.4043	0.4412	0.4910	0.5361	0.4698	0.4578	0.4835	0.4349



Online Resource 2 Calculation of arena wall height

In order to calculate the height of the arena's wall necessary to enable colour vision and disable sole perception via green contrast the following equation was applied: $h = g/(2*\tan(\alpha/2))$. (h = height of the arena; g = diameter of stimulus; α = visual angle).



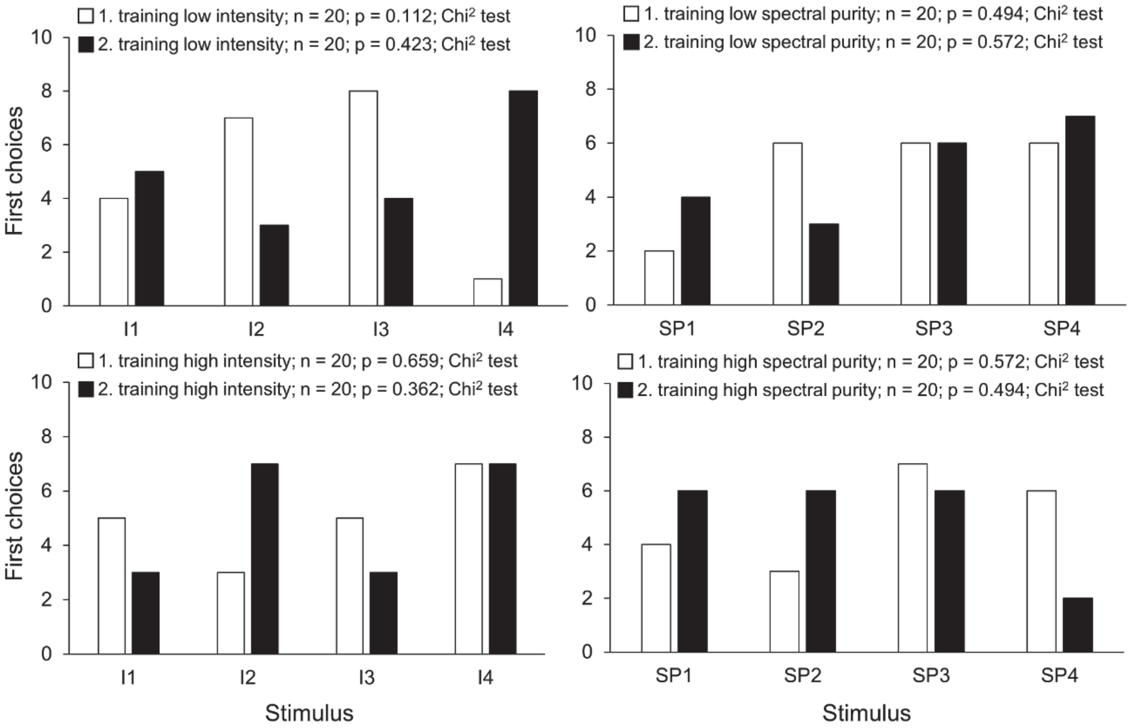
Online Resource 3 Experimental setup

(A) Testing arena with an UV-transmitting Feeder in its centre. (B-C) Training situations (as an example) for high and low spectral purity that were conducted consecutively separated by a test run. The same procedure was conducted for the stimuli with manipulated intensity. (D) Exemplary test arrangement with eight stimuli (two of each stimulus SP1-SP4 or I1-I4). (E-F) Foraging worker of *A. mellifera* and *T. carbonaria* on a stimulus.

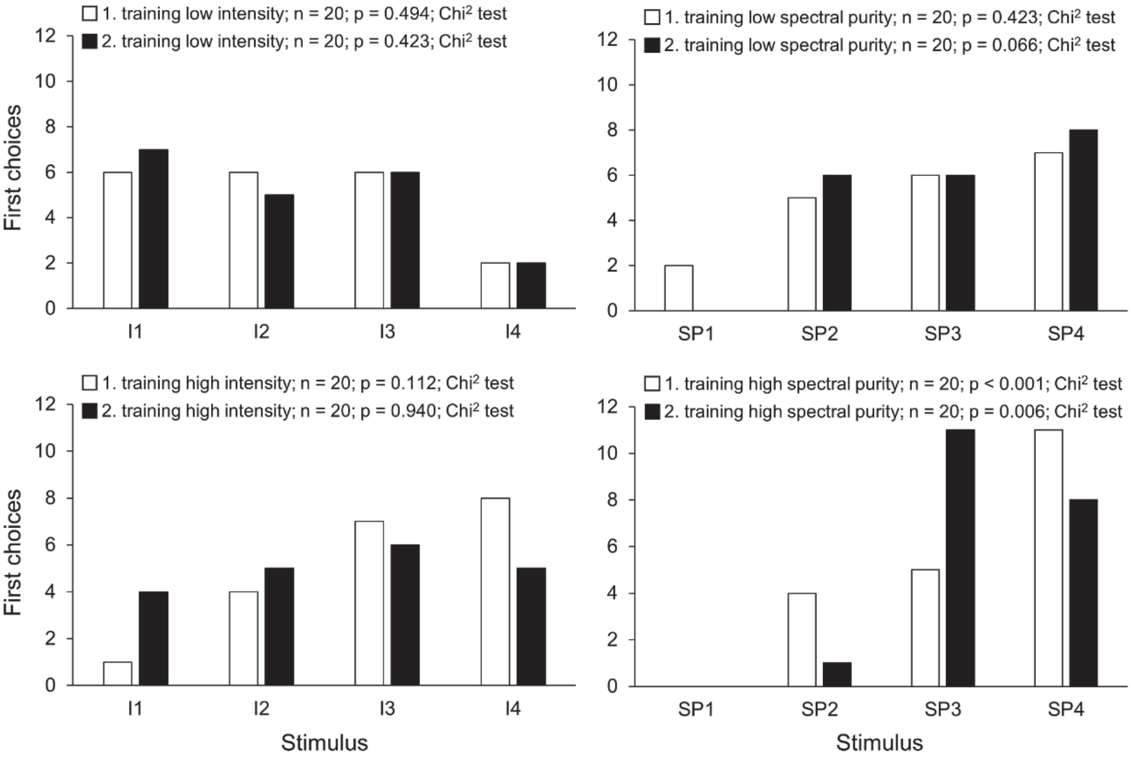
Online Resource 4 Results of Z-transformation according to Fisher

T1 = first training; T2 = second training; rho = Pearson's correlation coefficient; min. = lower limit of confidence interval; max. = upper limit of the confidence interval

	T1	rho	min.	max.	T2	rho	min.	max.	*p*-value
T. carbonaria	SP1	0.9234	1.1361	2.0869	SP1	-0.9487	-2.2938	-1.3431	<0.001
	SP4	0.9909	2.2169	3.1676	SP4	0.7746	0.5563	1.5071	<0.001
	I1	0.8889	0.9416	1.8923	I1	-0.5758	-1.1316	-0.1808	<0.001
	I4	0.2828	-0.1846	0.7661	I4	0.7379	0.4704	1.4211	0.028
A. mellifera	SP1	0.9311	1.1915	2.1422	SP1	0.8627	0.8286	1.7793	0.145
	SP4	0.9891	2.1266	3.0774	SP4	0.9932	2.3668	3.3175	0.242
	I1	-0.8706	-1.8108	-0.8601	I1	-0.8581	-1.7615	-.08108	0.443
	I4	0.9106	1.0555	2.0062	I4	0.8367	0.7346	1.6853	0.175



Online Resource 5 First choices of *T. carbonaria*



Online Resource 6 First choices of *A. mellifera*

CHAPTER 6

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A comparative study of food source selection in stingless bees and honey bees

Koethe S, Fischbach V, Banysch S, Reinartz L, Hrncir M, Lunau K

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A comparative study of food source selection in stingless bees and honey bees

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Abstract

In social bees, the choice of food sources is based on several factors, including scent marks deposited by con- and heterospecifics, colour and location. Here, we used two experimental setups, in which two species of stingless bees, *Melipona subnitida* and *Plebeia flavocincta*, and the Western honey bee, *Apis mellifera*, were tested to determine whether workers chose food sources according to 1) scent cues deposited by conspecifics, 2) the colour of a food source, 3) the trained location or 4) the proximity of a food source to the hive. All three species preferred the scent marked over an unmarked feeder that was presented simultaneously, but *M. subnitida* showed a weaker preference compared to the other species. When trained to blue feeders all bees preferred blue but *A. mellifera* showed the strongest fidelity. The training to yellow feeders led to less distinct colour choices. Only workers of *M. subnitida* mostly orientated at the training position and the close proximity to the nest. The disparity between the species corresponds to large differences in body size. Smaller bees are known for reduced visual capabilities and might rely less on visual parameters of the target such as colour hue, saturation or brightness but use scent cues instead. Moreover, the dim-light conditions in forest habitats might reduce the reliability of visual orientation as compared to olfactory orientation.

Keywords stingless bees, honey bees, scent marks, colour, location, recruitment

INTRODUCTION

While foraging, bees use visual and olfactory cues to find and select food sources. To detect flowers from a distance, innate or learned preferences are important factors (Lunau and Maier 1995; Dyer et al. 2016). Primarily, a forager's choice is biased by innate preferences for particular colours, shapes, and odours (Menzel 1967; Giurfa et al. 1995; Lehrer et al. 1995; Lunau et al. 1996; Gumbert 2000; Biesmeijer and Slaa 2004; Raine and Chittka 2007). These innate preferences differ between species. In several experiments, preferences for specific hues and saturation of colours could be found for honey bees and bumble bees (Lunau 1990; Giurfa et al. 1995; Lunau et al. 1996; Rohde et al. 2013; Papiorek et al. 2013), while stingless bees sparsely show preferences for colour hue or saturation (Spaethe et al. 2014; Dyer et al. 2016; Koethe et al. 2016, 2018).

With increasing foraging experience, initial individual preferences may be either consolidated or modified through associative learning (Gumbert 2000; Sánchez et al. 2008; Roselino et al. 2016). For instance, species-specific chemical footprints deposited by bees while landing on and manipulating flowers indicate the recent presence of a forager to subsequent visitors (Hrncir et al. 2004; Jarau et al. 2004; Eltz 2006; Saleh and Chittka 2006; Witjes et al. 2011). An initial attraction towards the familiar scent of conspecifics (Schmidt et al. 2005) may be reinforced when individuals learn to associate the footprints with high reward levels, or reversed when scent marks indicate depleted flowers (Saleh and Chittka 2006; Roselino et al. 2016).

Learning and memory play a major role in bee foraging, enabling the repeated visit to sustainable food sources (Breed et al. 2002; Reinhard et al. 2004, 2006; Jesus et al. 2014), flower constancy (Free 1963; Biesmeijer and Toth 1998; Slaa et al. 1998, 2003), and the discovery of new patches of known food plants (Biesmeijer and Slaa 2004). In addition to memorizing scent and location of resources (Reinhard et al. 2004, 2006), bees learn both colour and position of landmarks, which facilitates the orientation towards food sources and the nest (Cartwright and Collett 1983; Cheng et al. 1986, 1987; Chittka et al. 1995; Menzel et al. 2005). However, species differ concerning their learning ability (Pessotti and Lé'Sénéchal 1981; Mc Cabe et al. 2007), which might be associated with differences in life-history and ecological traits between bee species, such as longevity of individuals (Ackerman and Montalvo 1985), the degree of floral specialisation (Cane and Snipes 2006), and food niche-breath (Biesmeijer and Slaa 2006).

In eusocial bees, including the stingless bees (Meliponini), bumble bees (Bombini), and honey bees (Apini), food source selection is not only based on individual foraging preferences, but relies to a large extent on social information. On their return to the nest, foragers transmit olfactory and gustatory information about the exploited food source to nestmates, which biases the subsequent food choice of the receivers (Farina et al. 2005, 2007; Mc Cabe and Farina 2009). Moreover, returning foragers of many species announce the existence of lucrative food sources through thoracic vibrations (stingless bees: Lindauer and Kerr 1958; Esch et al. 1965; Barth et al. 2008; Hrncir and Barth 2014; bumble bees: Schneider 1972; honey bees: Esch 1961; Waddington and Kirchner 1992; Hrncir et al. 2011). Inactive individuals may use these mechanical signals for their decision of whether to engage in foraging or to remain in the nest. In addition, foragers of some eusocial bee species guide the recruits to the location of the exploited food patch. Honey bees (all species) use an elaborated dance language (waggle dance) communicating information about distance, direction, and quality of foraging sites (von Frisch 1967; Dyer 2002). Stingless bees (few species), in contrast, lay polarized trails of species-specific pheromone marks that guide recruits with high precision towards the goal (Lindauer and Kerr 1958; Schmidt et al. 2003; Nieh et al. 2004; Barth et al. 2008; Jarau 2009). At the food patch, foraging choices are influenced by field-based social information, like olfactory footprints and the visual presence of con- or heterospecific foragers (Slaa et al. 2003). Depending on the composition of the foraging community at the food patch, these passively provided cues may cause local enhancement or local inhibition (Slaa and Hughes 2009). Thus, food source selection in eusocial species is based on a complex interplay between individual preferences and social information.

Differences between social bee species with regard to ecological (habitat, food niche), physiological (learning ability, visual capacity, colour vision), and behavioural features (innate preferences, foraging strategy, recruitment mechanism) may result in differences concerning the parameters used in foraging decisions. In the present study, we investigated the food source selection by two stingless bee species, *Melipona subnitida* and *Plebeia flavocincta*, and the Western honey bee (*Apis mellifera*). Since stingless bees show only weak preferences for colours compared to other bee species (Dyer et al. 2016; Koethe et al. 2016, 2018), alternative parameters could be of importance for foraging choices. Of particular interest were the roles of scent marks (olfactory footprints), the colour, and the location of a food source. The aim was to analyse whether the three investigated social bee species use these parameters differently during colony foraging processes.

MATERIAL AND METHODS

Study site and bee species

The foraging behaviour of the stingless bee species was investigated at the Brazilian Federal University at Mossoró (Universidade Federal Rural do Semi-Árido), located in the Brazilian tropical dry forest, the Caatinga. For our experiments, we used two stingless bee species native to the study region, *Melipona subnitida* (six colonies) and *Plebeia flavocincta* (one colony) (Zanella 2000; Imperatriz-Fonseca et al. 2017). Colonies of these species were kept in wooden nest-boxes at the university's meliponary (Meliponário Imperatriz) and were freely foraging. *Melipona* species are known to mark food sources with olfactory footprints (Jarau 2009; Roselino et al. 2016). For *P. flavocincta* no specific information concerning scent communication is available so far (Aguilar et al. 2005). However, given that all bee species studied to this moment deposit chemical footprints at food sources (Goulson et al. 1998; Eltz 2006; Yokoi et al. 2007; Jarau 2009; Witjes et al. 2011), scent cues can also be postulated in this meliponine species.

The foraging behaviour of the Western honey bee, *Apis mellifera*, was studied at the botanical garden of the Heinrich Heine University Düsseldorf, Germany. Foragers of five nests were trained to participate in the experiment. *Apis mellifera* is known for marking food sources directly (Giurfa & Núñez 1992).

Training phase

For all tests and bee species the training was identical. Workers were trained to mass feeders offering sugar solution (50 % weight on weight). The training to the mass feeders started at the respective nest's entrance. Mass feeders were affixed to tripods in order to maintain mobility. After more than 10 workers regularly foraged at the mass feeder, it was moved in short steps (~1 m) away from the nest until a distance of 15 m or 17 m was reached. Once at the final feeding site (site 1 = 15 m; site 2 = 17 m), the mass feeder was replaced by a coloured gravity feeder (10 cm diameter, 5 cm height) that was used during the experiment. The gravity feeders were either blue (edding permanent spray RAL5010 enzianblau, edding International GmbH, Ahrensburg, Germany) or yellow (only for the colour test; edding permanent spray RAL 1037 sonnengelb, edding International GmbH, Ahrensburg, Germany). The colours were measured using spectrometer analysis (USB4000 miniature fibre optic spectrometer, Ocean Optics GmbH, Ostfildern, Germany) at an angle of 45° using a UV-NIR deuterium halogen lamp (DH-2000-BAL, Ocean Optics GmbH), which was connected to the spectrometer by a UV-VIS fibre optic cable (Ø 600 µm, QR600-7-UV 125 BX, Ocean Optics GmbH). To calibrate the spectrometer, a black standard (black PTFE powder, Spectralon diffuse reflectance standard SRS-02-010, reflectance factor of 2.00 %, Labsphere, Inc. North Sutton, USA) and a white standard (white PTFE powder, Spectralon diffuse reflectance standard SRS99-010, reflectance factor of 99.00 %, Labsphere, Inc. North Sutton, USA) were used (Fig. 1). After the workers accepted the coloured gravity feeder (henceforth "feeder"), a training period of 30 minutes started in which the bees were allowed to forage *ad libitum* (approximate number of foragers during training phase*: M. subnitida* ≈ 10 individuals; *P. flavocincta*, *A. mellifera* ≈ 30-50 individuals). Workers were not marked during the training to keep the disturbance at the feeder to a minimum. Hence, no discrimination between experienced and inexperienced workers was possible.

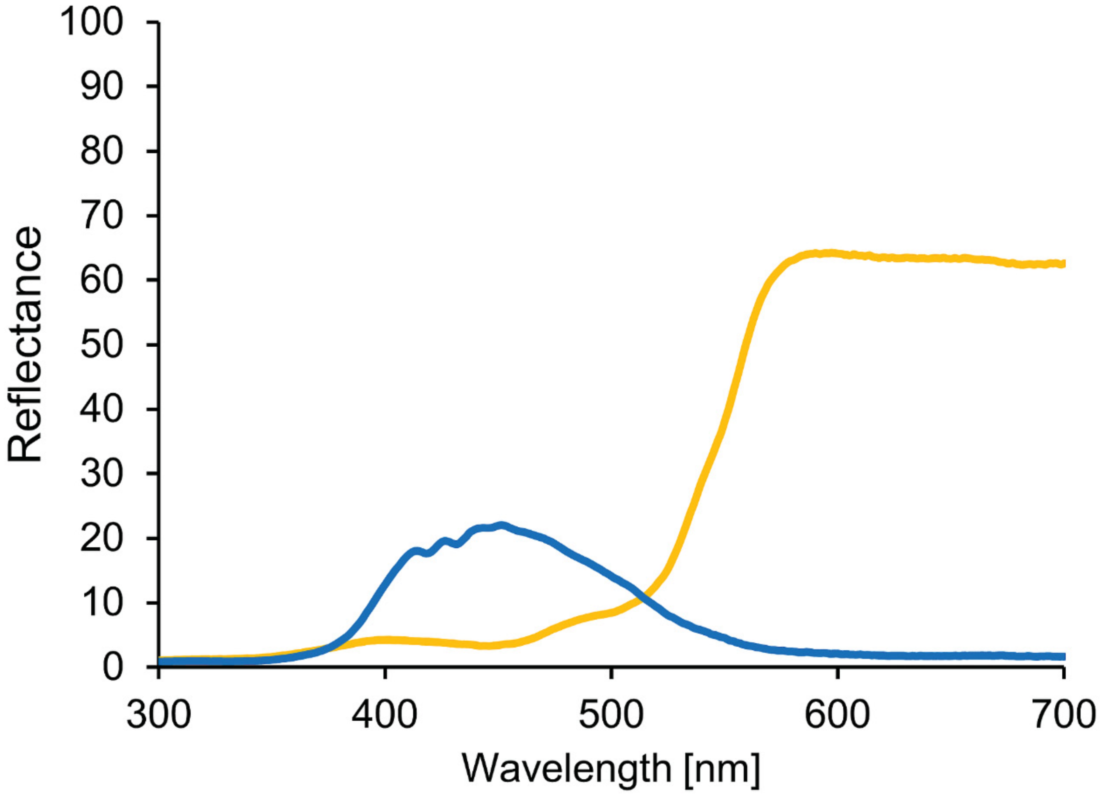


Fig. 1 Spectral reflectance curves of coloured feeders

Testing phase

Testing the impact of scent marks

We conducted experiments investigating the influence of scent marks deposited at the training feeder on the choice behaviour of foragers. For this experimental series, we used only blue-coloured feeders. After the initial training phase, we offered the incoming bees both the training feeder (scent-marked) and a clean blue-coloured feeder (unmarked), one at each feeding site (Table S1). In total, we performed three trials of this experimental series with each bee species. A trial consisted of three 5-minute test phases intermitted by 30-minute training phases (SM1-SM2; Table S1), switching the feeder positions in pseudo-randomized order. The three different bee species (*A. mellifera*, *M. subnitida* and *P. flavocincta*) were tested separately. Workers that visited the feeder were either marked with nail polish on their first visit (*A. mellifera* and *M. subnitida*), or caught after landing (*P. flavocincta*) and released at the end of the respective 5-minute test interval. Workers were allowed to participate in all three tests. To avoid pseudo-replication

(*A. mellifera*, *M. subnitida*), only the first landing of an individual in each test was considered for the analysis. During the third test, all foragers were captured and sacrificed to avoid pseudo-replication.

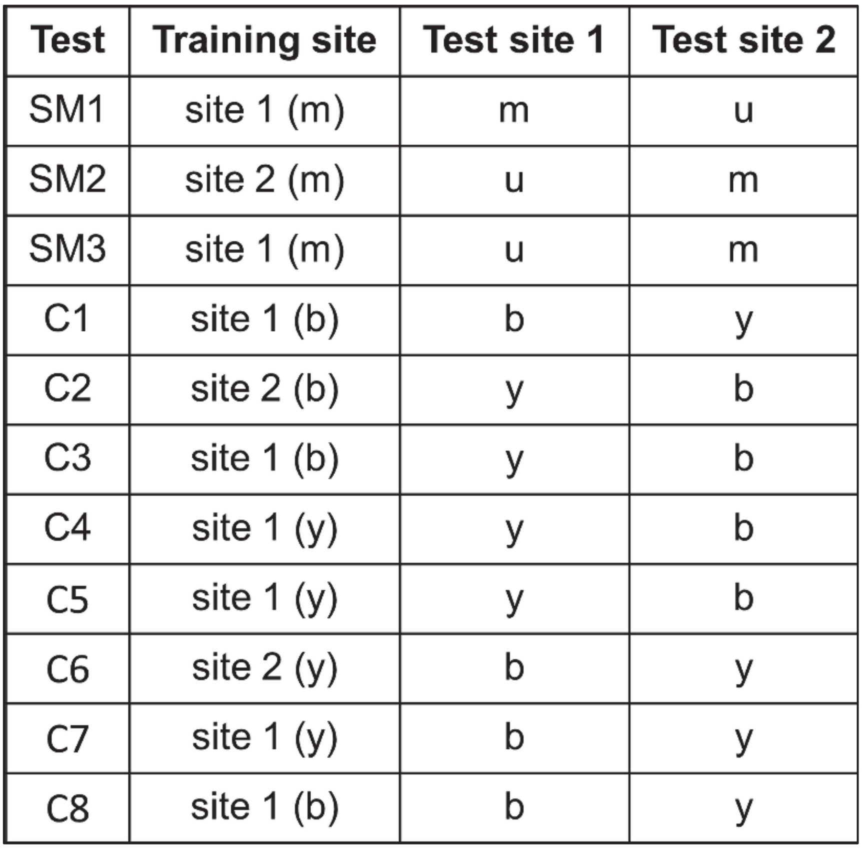
Testing the impact of colour

In the second experimental series, we investigated the impact of colour on the choice of food sites by workers. After the initial training phase (training feeder either blue or yellow; Table S1), the training feeder was removed, and we offered the incoming bees a blue- and a yellow-coloured feeder, one at each feeding site (Table S1). We performed two trial series, each comprising four 5-minute test phases intermitted by 30-minute training phases, switching feeder positions in a pseudo-randomized order (Table S1). In trial series 1 (C1-C4; Table S1), bees were trained on blue feeders in the first three training phases and on a yellow feeder in the fourth (training to blue, retraining to yellow). In trial series 2 (C5-C8; Table S1), foragers were trained on yellow feeders during three training phases and on a blue feeder in the last training phase (training to yellow, retraining to blue). For the test phases we used alcohol-cleaned feeders to eliminate the influence by any potential scent marks. Both trial series were repeated three to five times with different individuals. The bee species (*A. mellifera*, *M. subnitida* and *P. flavocincta*) were tested separately and workers that visited the feeder were either marked with nail polish (*A. mellifera* and *M. subnitida*), or caught after landing on a feeder (*P. flavocincta*) and released at the end of the respective 5 test phase. To avoid pseudoreplication (*A. mellifera*, *M. subnitida*), only the first landing of an individual in each test was considered for the analysis. During the fourth test, all workers were captured and sacrificed.

Testing the impact of location

To test whether bees visited the feeding site closer to the nest (site 1, 15 m) more often than the farther feeding site (site 2, 17 m) the results of all above described tests (scent marks and colour) were pooled based on the feeder’s position. Moreover, the visits of workers to the training position were pooled and compared with visits to the alternative feeder position.

Tab. 1 Position of feeders in training and test phase. The order of tests was pseudo-randomized to ensure no influence of test order on the decisions of workers. Two experimental trials were conducted comprising four choice experiments each. C1 - C4 are the tests which focused on blue colour, while the tests C5 - C8 focused on yellow colour. SM 1 - 3 = are tests which analysed the impact of scent marks; m = marked feeder; u = unmarked feeder; C 1 - 8= are the tests analysing the impact of colour; b = blue; y = yellow; site 1 = 15 m distance to the hive; site 2 = 17 m distance to the hive



Test	Training site	Test site 1	Test site 2
SM1	site 1 (m)	m	u
SM2	site 2 (m)	u	m
SM3	site 1 (m)	u	m
C1	site 1 (b)	b	y
C2	site 2 (b)	y	b
C3	site 1 (b)	y	b
C4	site 1 (y)	y	b
C5	site 1 (y)	y	b
C6	site 2 (y)	b	y
C7	site 1 (y)	b	y
C8	site 1 (b)	b	y

Statistics

All data were tested using multiple logistic regression to analyse the impact of scent marks, colour, training position and distance. The analysis was conducted with the statistical program R (R Development Core Team 2008).

Results

In the first experimental series (influence of scent marks), foragers of all three bee species significantly preferred the previously visited training feeder over the clean feeder (Fig. 2; *M. subnitida*: n = 239, p < 0.001; *P. flavocincta*: n = 355, p < 0.001; *A. mellifera*: n = 303, p < 0.001).

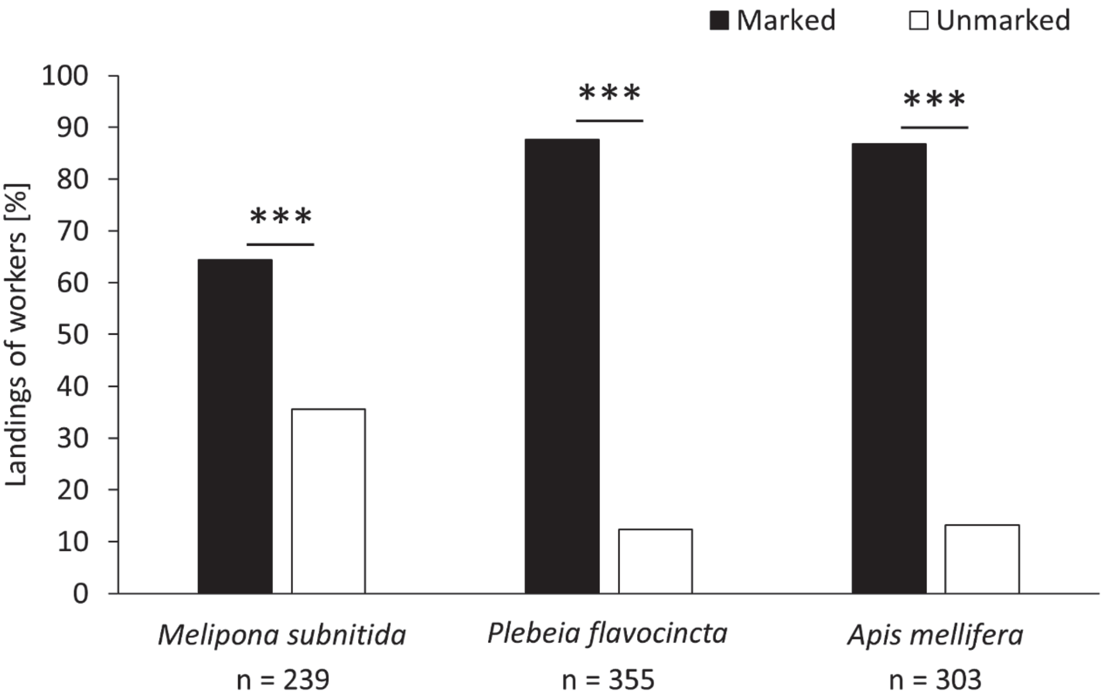


Fig. 2 Landings of workers on a scent-marked and an unmarked feeder. A multiple logistic regression analysis was used for statistical analysis (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$)

In the second experimental series, we investigated the influence of colour on the feeder choice by the three bee species. After training to a blue-coloured feeder, all three species significantly preferred the blue feeder over the yellow feeder was (Fig. 3A; *M. subnitida*: n = 250, p < 0.001; *P. flavocincta*: n = 230, p < 0.001; *A. mellifera*: n = 538, p < 0.001). When these workers were retrained to forage on a yellow feeder during the last training phase, the two stingless bee species significantly preferred the yellow feeder while honey bee workers visited both colours equally (Fig. 3B; *M. subnitida*: n = 124, p = 0.007; *P. flavocincta*: n = 71, p < 0.001; *A. mellifera*: n = 278, p = 0.549). When workers were initially trained to a yellow-coloured feeder, only workers of *M. subnitida* preferred the yellow feeder significantly over the blue feeder during the test, while *P. flavocincta* did not distinguish between the two colours, and *A. mellifera* even preferred the blue feeder (Fig. 3C; *M. subnitida*: n = 199, p = 0.005; *P. flavocincta*: n = 303, p = 0.488; *A. mellifera*: n = 556, p < 0.001). Retraining to a blue feeder in the last training phase lead to a significant preference of the blue coloured feeder in all three species (Fig. 3D; *M. subnitida*: n = 52, p = 0.003; *P. flavocincta*: n = 61, p = 0.010; *A. mellifera*: n = 213, p < 0.001).

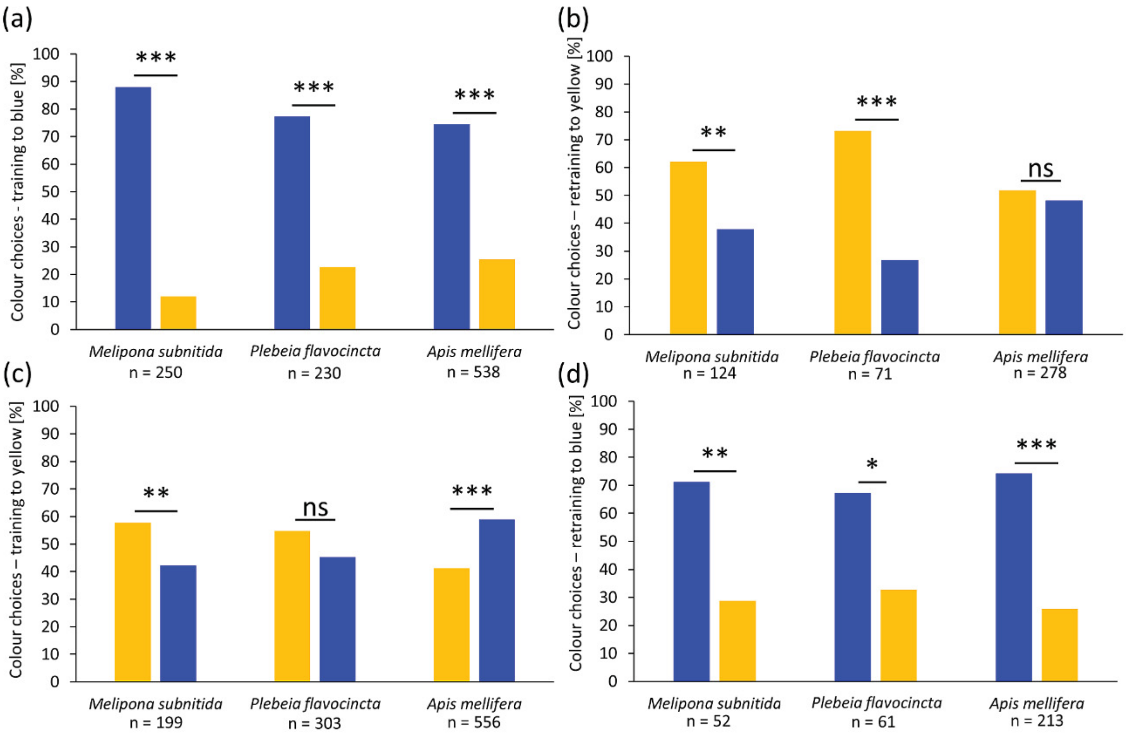


Fig. 3 Colour choices after training and retraining. The three tested bee species were trained to forage on either a blue feeder (A) or a yellow feeder (C). Furthermore, the workers were retrained to the opposite colour (B and D). A multiple logistic regression analysis was used for statistical analysis (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$)

When analysing the influence of the feeders' positions on the food source choice (pooled data from experimental series 1 and 2), we observed that *M. subnitida* visited the feeding site closer to the nest (site 1, 15 m) significantly more often than the farther site (site 2, 17 m) (Fig. 4A; n = 864, p = 0.023), while workers of *A. mellifera* significantly preferred the farther away feeding site (Fig. 4A; n = 1888, p = 0.003). Workers of *P. flavocincta* visited both feeding sites equally (Fig. 4A;

n = 1020, $p = 0.092$). Moreover, both stingless bee species visited the previous training site significantly more often, while *A. mellifera* did not differentiate the two feeding sites according to the previous training (Fig. 4B; *M. subnitida*: n = 864, $p < 0.001$; *P. flavocincta*: n = 1020, $p < 0.001$; *A. mellifera*: n = 1888, $p = 0.0744$).

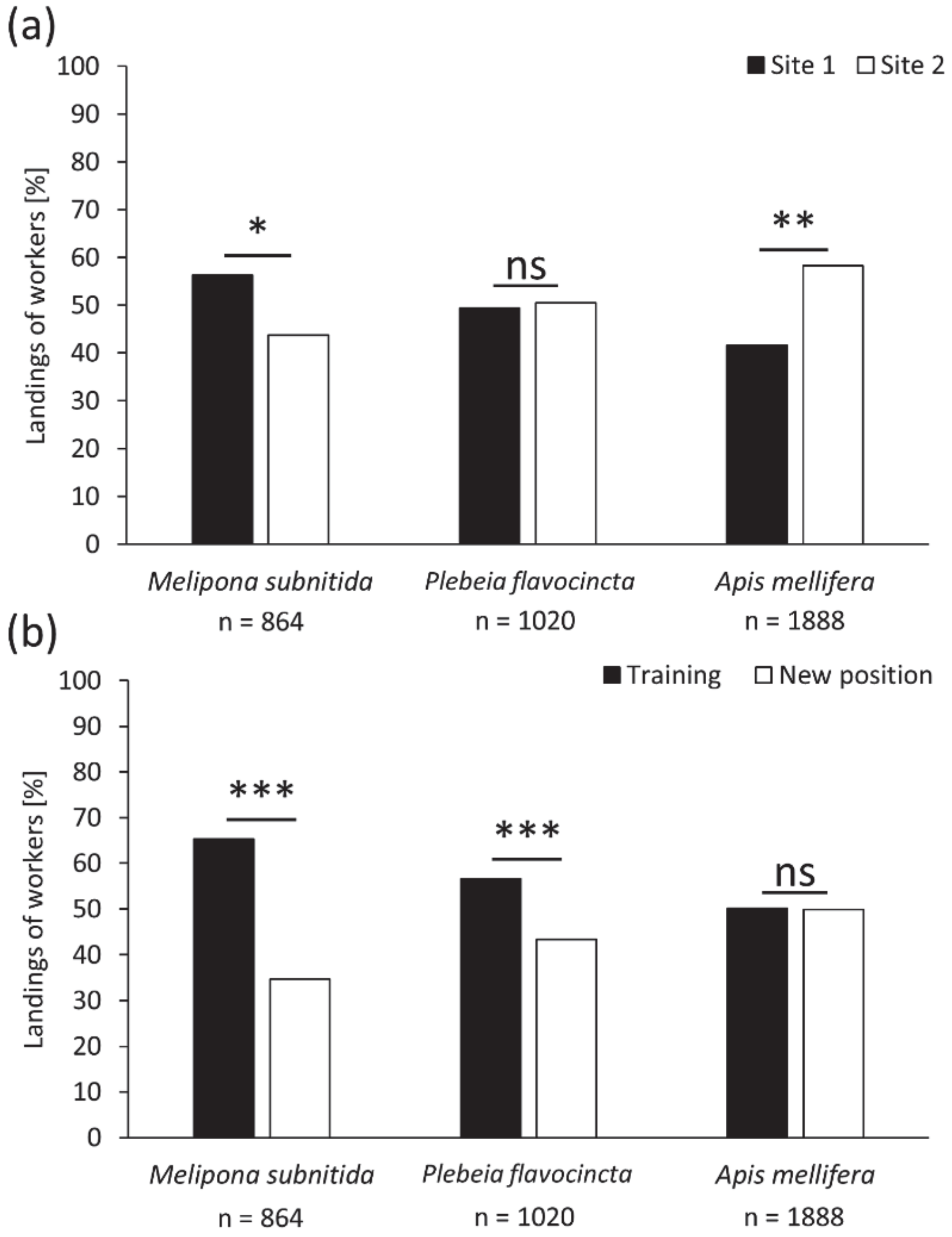


Fig. 4 Landings of workers depending on feeding site and position of training. (A) The number of landings at the feeding sites with 15 m (site 1) and 17 m (site 2) distance to the hive were compared (chi-square test of goodness-of-fit with Yates' correction; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). (B) The position to which the bees were trained (training) and the newly introduced position (new position) were analysed concerning the number of landings (multiple logistic regression analysis)

Discussion

With more than 500 described species, stingless bees (Meliponini) are the most speciose group of eusocial bees with very diverse characteristics regarding body size, colony size, nesting biology, brood cell arrangement, queen production, foraging strategies, and recruitment mechanisms (Michener 1974; Johnson 1983; Wille 1983; Engels and Imperatriz-Fonseca 1990; Roubik 2006, Barth et al. 2008; Michener 2013). Given this biological diversity, we can expect differences concerning the mechanisms of food source selection between species. In previous studies of colour preferences in stingless bees, the results varied among species. While three species of the genus *Melipona* chose colours poorly, *Tetragonula carbonaria* chose colours according to their hue and *Partamona helleri* showed similar colour choices as *Apis mellifera* preferring spectrally purer colours and blueish colour hues (Rohde et al. 2013; Dyer et al. 2016; Koethe et al. 2016, 2018).

Our study shows similarities and dissimilarities between the three tested bee species (*Melipona subnitida*, *Plebeia flavocincta*, *Apis mellifera*). Workers of *A. mellifera* orientated most strongly according to colours. The blue-coloured feeder was preferred in all tests with exception of the retraining to yellow, where the three species showed no depicted choice for one of the two colours. This is in accordance to previous studies showing that *A. mellifera* prefers blue colours over other colour hues (Giurfa et al. 1995; Horridge 2007). The two stingless bee species chose feeders according to their colours but rather preferred the feeder colour of the previous training. Only when initially trained to yellow they showed weak (*M. subnitida*) or no preferences for the trained colour (*P. flavocincta*). This preference for blue is in accordance with previous results of stingless bees, but also suggests that it is weaker in stingless bees than in honey bees (Dyer et al. 2016; Koethe et al. 2016). An explanation for less visually driven behaviour in stingless bees could be the size differences compared to honey bees. *P. flavocincta* reaches a body size of 3.6 – 4.1 mm, *M. subnitida* of 7.5 mm, and *A. mellifera* is the largest of the three species with 13 – 16 mm (Hrncir and Maia-Silva 2013; Maia-Silva et al. 2015; Imperatriz-Fonseca et al. 2017). Especially the size of the eyes, which is associated with body size, can impact the visual capacities of bees (Streinzer et al. 2016). *P. flavocincta* is rather small, consequently their eyes are also small leading to poorer visual capabilities and this could be an explanation why workers of *P. flavocincta* orientated more strongly towards scent marks than workers of the other two bee species that were more strongly influenced by colour.

Stingless bees and honey bees use scent cues to evaluate reward availability of food resources (Nuñez 1967; Butler et al. 1969; Ferguson and Free 1979; Free and Williams 1983; Corbet et al. 1984; Giurfa and Nuñez 1992; Giurfa 1993; Stout et al. 1998; Williams 1998; Stout and Goulson 2001). In this study, all three species showed preferences for the marked feeder over the unmarked one. *P. flavocincta* and *A. mellifera* chose the marked feeder consistently (~ 88 % of choices), while *M. subnitida* preferred the marked feeder, but visited it less frequently (~ 64 % of choices).

M. subnitida was the only species in the tests that visited the food site with shorter distance to the hive more frequently and was affected by the different trainings. It seems likely that *M. subnitida* orientates on location rather than on scent marks. Previous studies showed that species of the genus *Melipona* mark food sites directly and do not lay scent trails (Hrncir et al. 2004). In order to recruit new foragers it seems possible that *M. subnitida* relies strongly on piloting – leading new foragers from hive to food site during flight (Nieh et al. 2003). Foragers of *M. subnitida* could be observed to frequently arrive in small groups, while *A. mellifera* and *P. flavocincta* workers seemed more independent from each other. Scent marks play an important role for the communication of reward availability, but their impact on recruitment seems dependent on the specific strategy used by species (Free & Williams 1983; Corbet et al. 1984; Giurfa & Nunez 1992; Giurfa 1993; Stout et al. 1998; Stout & Goulson 2001; Schmidt et al. 2003). The attractiveness of scent marks, whether or not they were used for recruitment purposes, appears to be strong because scent-marked feeders were preferred by all three tested bee species. During the experiments workers foraged in groups and could be influenced by the presence of other individuals. An influence by social facilitation (Wilson 1971) could not be excluded during the experiments, but when comparing the results for choices of blue and yellow feeders, after the respective training, an influence solely by the presence of conspecifics seems unlikely.

Another aspect that can explain the diverse results for the three tested bee species could be their natural habitat. *M. subnitida* originates from the Caatinga, which is an open habitat, while *P. flavocincta* inhabits a spacious habitat that extends from the Caatinga to the Atlantic Rainforest, which is a densely vegetated forest (Imperatriz-Fonseca et al. 2017). Because of its domestication, the honey bee is widespread all over the world. It originates from diverse habitats of Europe, the Middle East and Africa. Open habitats are brightly illuminated, while forest habitats are characterized by dim-light conditions (Endler 1992). Based on the light conditions of their respective habitat, it appears to be possible that *M. subnitida* and *A. mellifera* rely to a greater extent on visual signals than *P. flavocincta* that encounters dim-light conditions and a less visually structured vegetation. In a densely vegetated habitat, scent marks could be a more reliable signal to guide foragers to a food source. Furthermore, temperate and sub-tropical regions experience more distinct seasons concerning weather conditions and the rhythm of flowering plants is directly influenced, while tropical and semi-arid regions have more steady weather conditions, but are challenging for their inhabitants because of high temperatures (Prado 2003; Zanella and Martin 2003; Machado and Lopes 2004; Maia-Silva et al. 2012, 2015). Social bee species that face seasonal variations mass-collect floral resources for provision of the hive (Ramalho 2004). These variations in floral resource availability could explain more distinct preferences for visual signals in honey bees when compared to tropical species, like *M. subnitida* and *P. flavocincta*, because only honey bees face strong seasonal variations (Michener 1974; Kleinert-Giovannini 1982; Roubik 1982, Seeley 1985).

In conclusion, the three tested bee species reacted vaguely similar to colour, scent marks, and location of food sources, but their main focus varies: While *A. mellifera* choose food sites according to both colour and scent marks, *M. subnitida* orientates on location and colour of food sites, and *P. flavocincta* relies mainly on scent marks. These variations are possibly based on different recruitment mechanisms (e.g. waggle dance of honey bees vs. piloting, excited movements, vibration, and scent mark deposition by stingless bees) or they could be the result of adaptations to the bees' respective habitat and obliged morphological constraints.

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Supplement

Online Resource 1 Comparisons of single tests. Results that do not differ significantly are marked in grey (chi-square test of goodness-of-fit with Yates' correction)

	Test	Number of workers on site 1	Number of workers on site 2	p-value (Chi^2)
Melipona subnitida	SM1	76	5	<0.001
	SM2	36	51	0.113
	SM3	44	27	0.059
	C1	70	6	<0.001
	C2	11	75	<0.001
	C3	13	75	<0.001
	C4	77	47	0.009
	C5	52	13	<0.001
	C6	36	34	0.906
	C7	35	29	0.532
	C8	37	15	0.004
Plebeia flavocincta	SM1	114	2	<0.001
	SM2	16	101	<0.001
	SM3	26	96	<0.001
	C1	5	84	<0.001
	C2	50	19	<0.001
	C3	44	28	0.077
	C4	19	52	<0.001
	C5	75	23	<0.001
	C6	45	64	0.085
	C7	69	27	<0.001
	C8	41	20	0.010
Apis mellifera	SM1	92	5	<0.001
	SM2	11	92	<0.001
	SM3	24	79	<0.001
	C1	114	15	<0.001
	C2	42	114	<0.001
	C3	80	173	<0.001
	C4	144	134	0.590
	C5	43	146	<0.001
	C6	119	126	0.701
	C7	62	60	0.929
	C8	158	55	<0.001

CHAPTER 7

SYNTHESIS

In field observations, stingless bees can be found to forage on flowers that are pollinated by birds (Roubik 1982). Usually these flowers are inconspicuous to bees because of their UV-absorbing red or UV-reflecting white colours (Porsch 1931; Raven 1972; Rodríguez-Gironés & Santamaría 2004; Lunau et al. 2011). Flowers that are categorized as ornithophilous usually offer high volumes of diluted nectar (Stiles 1981; Johnson & Nicolson 2007). Hummingbirds are important pollinators for many plant species and it was suggested that flower-visiting birds prefer floral colours that are not in the visual range of other pollinators like bees. Studies concerning colour preferences in hummingbirds disconfirmed this assumption because naïve hummingbirds do not prefer colours like UV-absorbing red or UV-reflecting white, which are achromatic to bees (Lunau et al. 2011). Only experienced hummingbirds showed preferences for red colours and, therefore, it is assumed that hummingbirds avoid the competition with bees by visiting flowers they can detect more easily than bees (Bergamo et al. 2016). If stingless bees are able to detect flowers that are usually pollinated by birds they possibly use colour parameters other than hue or saturation, but green contrast or brightness to find rewarding flowers. Light conditions in tropical habitats are probably too unsteady to provide reliable foundation for colour discrimination and detection, so that strongly rooted colour preferences cannot assure foraging processes of stingless bees (Endler 1993). Furthermore, the eye size of bees is critical for acuity and spatial resolution in bees and correlates with body size, which in stingless bees is usually smaller than in other social bee species (Streinzer et al. 2016).

Aim of this dissertation was to investigate colour preferences of stingless bees and compare these results with published and collected data for honey bees and bumble bees to compare colour preferences of social bees. It has been discussed that floral colour evolved the same way in different regions of the world and that this similarity is based on colour perception of bees or *vice versa*, that colour perception of bees is impacted by floral reflectance spectra (Chittka & Menzel 1992; Chittka & Wells 2004; Raine & Chittka 2007; Dyer et al. 2012; Shrestha et al. 2013). Sensitivities of photoreceptors are highly conserved in hymenopterans leading to the assumption that colour perception and colour preferences in closely related species, like honey bees and stingless bees, could be similar (Briscoe & Chittka 2001).

First, colour preferences of naïve workers of the Australian stingless bee *Tetragonula carbonaria* were investigated (Chapter 2, Dyer et al. 2016). Like in honey bees and bumble bees, bluish colours were preferred by *T. carbonaria*, but the preference of honey bees and bumble bees for saturated colours, which was previously reported, could not be found in *T. carbonaria* (Rohde et al. 2013; Papiorek et al. 2013). A combination of saturation and green contrast correlated with the decision of workers, but saturation alone had no impact on the worker's choices. To investigate the impact of saturation and other important colour parameters (hue, brightness and green contrast) in more detail, a number of experiments were conducted (Chapter 3-5, Koethe et al. 2016, 2018). *Partamona helleri* was the only tested stingless bee species that exhibited the same preferences for bluish and saturated colours, as it is known for honey bees and bumble bees (Lunau 1990; Chittka et al.1994; Giurfa et al 1995; Lunau et al. 1996; Raine et al. 2006a; Rohde et al. 2013; Papiorek et al. 2013). The preference for bluish colours and saturated colours of bees appear reasonable in the context that both parameters are related to higher floral rewards (Giurfa et al. 1995; Raine & Chittka 2007; Kantsa et al. 2017).

A previous study found variability in colour choices among colonies of the same species (Raine & Chittka 2007). An explanation for the differences in choice behaviour among colonies of the same species could be competition avoidance. Colonies that reside at the same location share a common pool of food sources, but collect rewards on different flowers based on learned preferences. The stingless bee species that were tested in Brazil (*M. mondury* and *M. quadrifasciata*, Chapter 3; *M. bicolor* and *P. helleri*, Chapter 4) were located in close proximity to each other, and because of the proximity to each diverging learned colour preferences could lead to the avoidance of competition. Whether bee colonies that share a habitat split food sources like that and, thereby, gain different experiences and learned colour preferences is not clear yet. Slaa et al. (2003) analysed the foraging behaviour of different stingless bee species, which share a habitat, based on social information outside the nest. The aim was to analyse whether single foragers decide to land at food sources that are occupied by nestmates, conspecifics or heterospecifics or if they avoid food sources that are taken. Some species preferred to land on food sources that were already occupied by nestmates, while others avoid foraging nestmates and conspecifics. Some of the analysed species avoided heterospecifics, and other species visited food sources that were occupied by non-aggressive, small foragers more frequently, while large aggressive foragers were mostly avoided (Slaa et al. 2003).

The processing of colours is different between similar and dissimilar colours in bees (Dyer & Chittka 2004). Bees are able to discriminate colours with large differences more easily than colours with small differences (Giurfa et al. 1994; Lehrer 1999; Dyer & Chittka 2004). When using broadband stimuli with varying colour hues in the experiment of Dyer et al. (2016), missing preferences for spectral purity could be explained by differential colour processing of colour hues (Wyszecki & Stiles 1928; Giurfa et al. 1996, 1997; Spaethe et al. 2001; Kemp et al. 2015). Broadband stimuli simulate natural flower colours in a more accurate way than monochromatic or narrowband stimuli (Chittka et al. 1994; Arnold et al. 2010; Dyer et al. 2016). The principle of univariance states that colours can hardly be discriminated when only a single photoreceptor is involved (Rushton 1972; Garcia et al. 2015). The method that was used in the Chapters 3-5 is based on artist pigments that produce broadband stimuli (Koethe et al. 2016, 2018). The great opportunity that is given with these pigments is that the manipulation of single colour attributes – brightness and saturation – is quiet easy. By adding white or black pigments in defined amounts both parameters can be controlled. The pigments are available in many colour hues and can be mixed to create further hues. When measured with a reflectance spectrometer, the colours match naturally occurring floral colours. During the experiments of Chapter 3 – 5, the methods how to test workers advanced. In Chapter 4 individual workers had to choose colours based on dual choices of stimuli with either differing hue or differing values for intensity and spectral purity. Because workers were sometimes forced to make decisions between normally unattractive stimuli the results led to possibly more diverse preferences than naturally occurring. Another disturbance could be the association of intensity and spectral purity variations in Chapter 3 and 4. The impact of colour traits on each other is probably influencing bees’ choices. Therefore, by splitting intensity and spectral purity and offering all stimuli at the same time the experimental setting was improved and adjusted for interspecific comparison of colour preferences in bees (Chapter 5).

Regarding colour preferences, stingless bees showed very weak preferences for specific colour traits. While some species showed preferences for bluish colours (*T. carbonaria*, *P. helleri*, *M. mondury*), saturation was only preferred by *P. helleri* and brightness did not influence the bees choices in general.

The circumstance that stingless bee show weak colour preferences in comparison with other social bees raises the question what other influences drive food source selection in stingless bees. One characteristic of social bees that appears to play a major role in foraging is their communication. Honey bees have their elaborated dance language that is absent in bumble bees and stingless bees (von Frisch 1967; Dyer 2002). Stingless bees on the other hand have an elaborated olfactory communication system (Lindauer & Kerr 1960). By depositing pheromone signals or driven by involuntarily footprints stingless bees are able to communicate with each other and recruit nestmates very effectively (Biesmeijer & Slaa 2004; Jarau et al. 2004; Hrncir et al. 2004; Nieh 2004). Based on known facts about scent mark deposition on food sites, the focus of Chapter 6 was directed to scent marks. Surprisingly, honey bees were not only influenced by colour – they preferred blue over yellow feeders – but they also were influenced by scent marks of conspecifics. Presumably, scent marks of honey bee workers led to the preference as observed by previous studies in which rewarding food sources were marked by deposited pheromones from the Nasanov gland to recruit nestmates (Free 1987; Reinhard & Srinivasan). Stingless bees of the genera *Plebeia flavocincta* and *Melipona subnitida* predominantly orientated on scent marks, but *M. subnitida* was also influenced by the distance between hive and food site by preferring the closer food site. Both species learned to associate colours with food rewards but showed no prevailing preferences.

To conclude the findings of this dissertation, stingless bees show only weak preferences for bluish colour hues, while saturation is only a relevant colour trait for *P. helleri* and for *T. carbonaria* when associated with green contrast in different colour hues. Like in honey bees and bumble bees, brightness has no impact on colour choice. More than colour, scent marks deposited by conspecifics are driving food source selection in stingless bees.

Despite these differences between honey bees and stingless bees, stingless bees have proven to be effective pollinators in their respective habitat and for crops in the field and in green houses. The information collected for this dissertation and by other researchers in the past, present and future will help to understand the biology of stingless bees in a greater scope, to improve their conservation and to make them suitable substitutes for introduced honey bees in context of pollination services.

CHAPTER 8

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CHAPTER 9

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CHAPTER 10

APPENDIX

List of publications

Thesis is based on the following manuscripts:

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

Dyer AG, Boyd-Gerny S, Shrestha M, Lunau K, Garcia JE, **Koethe S** and Wong BBM

Journal of Comparative Physiology A (2016) 202: 603-613.

Colour is more than hue: preferences for compiled colour traits in the stingless bees *Melipona mondury* and *M. quadrifasciata*

Koethe S, Bossems J, Dyer AG and Lunau K

Journal of Comparative Physiology A (2016) 202: 615-627.

Spectral purity, intensity and dominant wavelength: Disparate colour preferences of two Brazilian stingless bee species

Koethe S, Banysch S, Alves-dos-Santos I and Lunau K

PLoS ONE 13(9): e0204663

A comparative analysis of spectral purity and intensity considering the colour choices of *Tetragonula carbonaria* and *Apis mellifera*

Koethe S, Reinartz L, Heard TA, Dyer AG and Lunau K

Manuscript under review in Journal of Comparative Physiology A

A comparative study of food source selection in stingless bees and honeybees

Koethe S, Fischbach V, Banysch S, Reinartz L, Hrncir M and Lunau K

Manuscript under review in Insectes Sociaux

The authors’ contribution

Chapter 2 - Dyer AG, Boyd-Gerny S, Shrestha M, Lunau K, Garcia JE, **Koethe S** and Wong BBM (2016) Innate colour preferences of the Australian native stingless bee *Tetragonula carbonaria* Sm.

Journal of Comparative Physiology A (2016) 202: 603-613.

Editing and revising manuscript

Chapter 3 - **Koethe S**, Bossems J, Dyer AG and Lunau K (2016) Colour is more than hue: preferences for compiled colour traits in the stingless bees *Melipona mondury* and *M. quadrifasciata*

Journal of Comparative Physiology A (2016) 202: 615-627.

Statistical analysis
Writing the manuscript

Chapter 4 - **Koethe S**, Banysch S, Alves-dos-Santos I and Lunau K (2018) Spectral purity, intensity and dominant wavelength: Disparate colour preferences of two Brazilian stingless bee species

PLoS ONE 13: e0204663

Study design
Spectrophotometric analysis
Behavioural experiments with bees
Statistical analysis
Writing the manuscript

Chapter 5 - **Koethe S**, Reinartz L, Heard TA, Dyer AG and Lunau K *Apis mellifera* and *Tetragonula carbonaria* respond differently to colour stimuli of varied intensity and spectral purity

Manuscript under review in Journal of Comparative Physiology A

Study design

Spectrophotometric analysis

Behavioural experiments with bees

Statistical analysis

Writing the manuscript

Chapter 6 - **Koethe S**, Fischbach V, Banysch S, Reinartz L, Hrncir M and Lunau K A comparative study of food source selection in stingless bees and honeybees

Manuscript under review in Insectes Sociaux

Study design

Behavioural experiments with bees

Statistical analysis

Writing the manuscript

I hereby confirm that the above-given information are correct

Prof. Dr. Klaus Lunau

Declaration of self-contained work

Herewith, I confirm that I composed the dissertation

“Colour preferences in stingless bees”,

single-handed without utilizing illegitimate resources. I used no other than the cited references and facilities. This work has not been previously handed in to another university and was not subject to miscellaneous examinations.

Düsseldorf, 19.03.2019

Sebastian Köthe