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Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision

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Flowering plants in Australia have been geographically isolated for more than 34 million years. In the Northern Hemisphere, previous work has revealed a close fit between the optimal discrimination capabilities of hymenopteran pollinators and the flower colours that have most frequently evolved. We collected spectral data from 111 Australian native flowers and tested signal appearance considering the colour discrimination capabilities of potentially important pollinators. The highest frequency of flower reflectance curves is consistent with data reported for the Northern Hemisphere. The subsequent mapping of Australian flower reflectances into a bee colour space reveals a very similar distribution of flower colour evolution to the Northern Hemisphere. Thus, flowering plants in Australia are likely to have independently evolved spectral signals that maximize colour discrimination by hymenoptera. Moreover, we found that the degree of variability in flower coloration for particular angiosperm species matched the range of reflectance colours that can only be discriminated by bees that have experienced differential conditioning. This observation suggests a requirement for plasticity in the nervous systems of pollinators to allow generalization of flowers of the same species while overcoming the possible presence of non-rewarding flower mimics.

Keywords: flower; bee; pollination; Australia; Gondwana

1. INTRODUCTION

In many flowering plant (angiosperm) species, the transfer of pollen from one flower to another is entrusted to animal vectors, such as insects and birds [1–3]. Typically, pollination vectors are attracted to flowers in search of floral rewards such as pollen and nectar, and in the process of visiting multiple flowers incidentally transfer pollen between compatible flowers. Plants typically attract and aid the orientation of important pollinators to their flowers by using relevant cues including olfaction [4], colour [5] and shape [6].

The relationship between angiosperms and animal vectors is very important. Plants that have rewarding flowers which are easily detected and discriminated will have an increased probability of distributing pollen to conspecifics, and thus successfully reproducing [5,7,8]. At the same time, animals that make correct foraging decisions will potentially collect more nutrition per unit time [9,10]. Visual ecology principles suggest that signal providers and/or signal receivers will evolve, within biological constraints, to optimize the efficiency of this biological partnership [1,11–14].

Insects are among the major pollinators of angiosperms. In particular, individuals of some hymenopteran species, such as honeybees and bumblebees, have a tendency to be 'flower constant', and will repeatedly visit one type of flower as long as these flowers continue to offer rewards [15,16]. It is probably that the reason why some individual pollinators exhibit flower constancy is a limitation on how working memory can learn and recall multiple flower types [15,17,18]. There are likely to be significant reproductive advantages for plants that can maintain flower-constant pollination vectors, since pollen is mainly delivered to conspecifics, rather than being randomly distributed, as would be the case for wind-pollinated angiosperms [15]. Thus, there are significant fitness benefits for angiosperms that have flowers which are easily discriminated by flower-constant pollinators.

It is known from both electrophysiological recordings [19] and behavioural testing [20] that honeybees have trichromatic colour vision based on ultraviolet- (UV), blue- and green-sensitive photoreceptors. This distribution of colour receptors is highly conserved in most other hymenopteran insects and is derived from a basal visual system that predates the evolution of angiosperms [19,21,22]. Colour discrimination should be optimal at wavelengths closest to the position where spectrally different photoreceptors overlap [19,23]. Thus, trichromatic hymenopteran pollinators are likely to have best discrimination for wavelengths close to 400 and 500 nm [19], and behavioural experiments on free-flying honeybees have confirmed this theory [24].

In a study that explored the potential ecological implications of pollinator vision on the colours of flowers that evolved in the Middle East (Israel), a very close fit was observed between the regions of the electromagnetic spectrum, where bees best discriminate colour information (400 and 500 nm), and the 'inflection points' at which flower reflection curves show the largest changes

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in the quantity of reflected radiation [25]. Interestingly, investigations in Israel have yielded one of the earliest fossil pollen records of angiosperms in the Hauterivian (*ca* 133 Ma) [26]. Palynological records from southern England also reveal evidence for the appearance of angiosperms around this time [27]. Studies comparing the distribution of flower colours suggest that visual ecological constraints from hymenopteran trichromats have been a major influence on angiosperm evolution throughout the Middle East and Europe [25,28,29]. Importantly, this evolution of flower colours has not been a coevolution as hymenopteran vision is phylogenetically ancient and predates the evolution of angiosperms [22].

The geological isolation of the Australian continent makes it an interesting target for studying angiosperm flower colour evolution. Australia has a very distinctive bee fauna, with emphasis on species of the family Colletidae, and relatively few Apidea; the latter representing less than 15 per cent of the known Australian species [30]. Importantly, Australia has been separated from other major continental land masses since at least the end of the Eocene epoch (*ca* 34 Ma) [31–35], and endured a period of isolation before coming into contact with scattered southeast Asian terranes in the Miocene, *ca* 25 MA [32]. While there is evidence that floristic interchange between the Northern and Southern Hemispheres did occur during the Mid- to Late Cretaceous, plant groups extending to each hemisphere readily differentiated into discrete provincial taxa [36]. The high-latitude Mesozoic position of Australia, its subsequent isolation and later northward drift have resulted in a high degree of endemism in the continent's flora (*ca* 6% of families, 22% of genera and over 80% of species are endemic [37]) with many lineages extending back to the Paleogene or Cretaceous [38]. It currently remains unclear, however, to what extent the evolution of flower colours on the Australian continent may have been shaped by the colour discrimination capabilities of hymenopteran pollinators, as has occurred in the Northern Hemisphere.

An important, related issue in the context of understanding flower evolution is the degree to which individual insects generalize similar colours [39,40]. Studies in honeybees [41–43], bumblebees [44–47], hawkmoths [48] and ants [49] reveal that individual insects learn perceptually similar colours very differently, depending on either absolute conditioning (learning a target colour in isolation), or differential conditioning (a target colour is linked to a reward while a distractor colour contains no reward). Differential conditioning leads to a significantly higher capacity to make fine colour discriminations [41,43–45,48,49]. Currently, the reason why insect pollinators demonstrate the behavioural plasticity to learn a target colour in different ways is unclear. One hypothesis is that pollinators initially need bandwidth to accept signals resulting from the natural variability in plant flower pigments [50]; but in a situation in which bees may encounter similarly coloured non-rewarding flowers, their visual system may need to have the ability to fine tune its responses to maximize the collection of nutrition [45,50,51]. These theoretical considerations suggest that flower variability from conspecific plants should lie within the range of colorimetric distances that pollinators can discriminate following differential

conditioning, but otherwise generalize if receiving absolute conditioning.

Here, we test whether the evolution of flower colours in Australia fits the regions of the electromagnetic spectrum for which hymenopteran colour vision enables the best level of discrimination. We then use the dataset to understand the extent to which the degree of variability in colour signals produced by particular plant species matches the range of colour discrimination and generalization that has been observed in behavioural studies of important angiosperm pollinators.

2. MATERIAL AND METHODS
(a) *Is there a link between hymenopteran vision and Australian floral coloration?*
(i) *Data collection*
Australian native flowers were collected from Maranoa Gardens, Melbourne, Australia. Maranoa Gardens maintains a diverse collection of species from all over the continent. Species held in the collection are not selected on the basis of flower colour, but are selected by botanists to represent the diversity of Australian plants. Data collection was once per month from May 2009 to January 2010. During data collection, plants were chosen on the basis of a plant having more than three flowers present; otherwise plant selection was randomized. A UV photograph was taken of a flower from each plant using a digital UV camera (Fuji Finepix Pro S3 UVIR-modified charge-coupled device for UV imaging and fitted with a 105 mm f4.5 quartz UV-Nikkor lens and optically polished Baadar U-filter (325–369 nm half band width)) with calibrated UV-visible grey scales [52]. As UV rays are typically invisible to the human eye [53], this photographic representation enabled any different UV-reflectance areas of the flower to be identified and then measured with a spectrophotometer [54]. The spectral reflection functions of flowers were measured from 300 to 700 nm using a spectrophotometer (S2000) with a PX-2 pulsed xenon light source attached to a PC running SPECTRA SUITE software (Ocean Optics Inc., Dunedin, FL, USA) and calibrated against a UV reflecting white $BaSO_4$ standard (Ocean Optics). A total of 111 plant species were sampled, each with three replicates. For data management, flower spectra will be contributed to the open access web portal Floral Reflectance Database to allow subsequent meta-analyses of flower reflectance data [55].

(ii) *Spectral measurement analyses*
Spectral data of flower reflectances were analysed using a previously established methodology, which has already shown that honeybee colour discrimination closely fits angiosperm colours that have evolved in the Northern Hemisphere [25,28]. For colours to be best discriminated by a visual system, the reflectance curves should rapidly change in the parts of the electromagnetic spectrum where spectrally different photoreceptors overlap [24,25,56]. We thus quantified the occurrence of inflection points where there was a change of greater than 20 per cent reflectance of radiation in less than 50 nm of the spectrum. The midpoint of a particular inflection point was determined within 10 nm bins, which allowed for the quantification of the wavelength at which spectral curves changed [25]. The data of the frequency of inflection points were plotted versus wavelength (λ) and compared with an inverse $\Delta\lambda/\lambda$

function that quantifies the regions of the visual spectrum in which honeybee vision can best discriminate spectral information [24].

(iii) *Colorimetric analyses*

Colorimetric techniques allow analyses of how flower reflectance curves are processed by the visual system of an animal. In this study, we used a hexagon colour space [57] to represent the distribution of flower colours that have evolved in Australia considering hymenopteran trichromat vision. The hexagon colour model was used in relevant previous studies [25,28], and makes no specific assumptions about colour opponent channels so is currently the most applicable general model of hymenopteran colour vision [57]. As mentioned previously, current evidence is that the photopigments underlying trichromatic vision in hymenopteran species are highly conserved, including for bee families native to Australia, and the photopigments are thus derived from a basal visual system that predates the evolution of angiosperms [21,22]. It is thus possible to model Australian bee colour perception using hymenopteran trichromatic models [58]. We modelled hymenopteran vision with spectral sensitivity peaks at 350 nm (UV), 440 nm (blue: B) and 540 nm (green: G) [21,22] using a vitamin A1 visual template [21,59–61].

For the colour hexagon model, the relative amount of radiation absorbed by each of the photoreceptors P (UV, blue (B), green (G)) was calculated by numerically integrating the product of photoreceptor absorption $S(\lambda)$, spectral reflectance $I(\lambda)$ and the illumination $D(\lambda)$ (equation (2.1)) at 10 nm steps from 310 to 650 nm. The variable K is used to normalize each of the photoreceptors to the illumination reflected from the background ([57,59]; equation (2.2)). The spectral quality of radiation was taken to be 6500 K, corrected for photon flux, to give a good match with typical daylight conditions for foraging insects [59,62]:

$$P(\text{UV}, \text{B}, \text{G}) = K \int_{310}^{650} S(\lambda) I(\lambda) D(\lambda) \, d\lambda \qquad (2.1)$$

and

$$K = \frac{1}{\int_{310}^{650} S(\lambda) I_{\text{B}}(\lambda) D(\lambda) \, d\lambda}, \qquad (2.2)$$

where $I_{\text{B}}(\lambda)$ is the spectral reflectance of the background of green foliage.

The transduction of photoreceptor absorption (P) into receptor excitations (E) is given by

$$E = \frac{P}{P+1}. \qquad (2.3)$$

The receptor excitations (E_{SWS}, E_{MWS} and E_{LWS}) were plotted on orthogonal axes, each of unit length, and the colour of a flower was represented by the sum of the three vectors [57]. Coding is performed by two unspecified colour opponent mechanisms (x and y) and the output is given in equations (2.4 and 2.5) [57]:

$$x = \sin 60^\circ (E_{\text{LWS}} - E_{\text{SWS}}) \qquad (2.4)$$

and

$$y = E_{\text{MWS}} - 0.5(E_{\text{LWS}} + E_{\text{SWS}}). \qquad (2.5)$$

Colour distance in the hexagon colour space can be determined by the Euclidean distance between loci [57].

These colorimetric values can be interpreted as perceptual distance using psychometric testing that has been conducted on bumblebees [50,63] and honeybees [64,65], the two main model systems for hymenopteran colour vision.

Australian native plant flower colour frequencies in colour space were determined with a radial grid of 10° sectors dissecting the distribution of colour loci, and the frequency of floral colour loci within each sector was counted as described in previous work [28].

(b) *Does variability between flowers of the same species explain why insect pollinators have behavioural plasticity for colour learning?*

Pollinator colour perception is dependent on individual experience (conditioning procedure) [41,43,44,48,50,51]. Using bumblebees as a model to map psychometric colour functions, and considering 70 per cent choices as the threshold for reliable recognition [24], it has been shown that discrimination can be divided into three cases: (i) colour distances less than 0.04 hexagon units are not reliably discriminated by bees, (ii) distances between 0.04 and 0.11 hexagon units are only discriminated if bees receive differential conditioning, and (iii) distances greater than 0.11 hexagon units are reliably discriminated even with absolute conditioning [50]. These three cases allow for the formulation of hypotheses about why the visual system of hymenopterans may have evolved the capacity for behavioural plasticity for colour discrimination.

H1: if the degree of variability in the pigmentation of flower colour for a particular plant species is less than 0.04 hexagon units then this variability is less than the perceptual threshold for bee colour vision. This case is a null hypothesis and would suggest that plasticity in pollinator colour discrimination is not linked to deal with the colour variability of plant flowers.

H2: if the degree of variability in the pigmentation of flower colour for a particular plant species is greater than 0.04 hexagon units but less than 0.11 hexagon units then this degree of variability in flower colour can only be discriminated by bees following differential conditioning. This case would suggest that bees generalize similar colours so long as flower stimuli present a reward (essentially a case of absolute conditioning), but if multiple non-rewarding flowers (e.g. non-rewarding mimics) were present in a foraging environment, an experienced forager can learn to make fine discriminations.

H3: if the degree of variability in the pigmentation of flower colour for a particular plant species is greater than 0.11 hexagon units, then this variability in flower colour is greater than the perceptual threshold for bee colour vision to reliably discriminate colours even with absolute conditioning.

Using this hypothesis-driven framework based on psychophysics testing, the colorimetry analyses method described above was used to determine the hexagon model colour difference between the data of the three flowers collected from each plant as sample 1 versus 2; 1 versus 3; and 2 versus 3 to produce one mean value of colour variability for each plant. This procedure was repeated for all 111 plants species, and overall colour variability was calculated as the mean ($\pm$ s.d.) of the 111 values to represent variation in natural flower coloration as perceived by bee pollinators.

3. RESULTS

(a) *Is there a link between hymenopteran vision and Australian floral coloration?*

Flower reflection curves measured with a spectrophotometer allow for the quantification of a flower's spectral 'signature'. Figure 1 shows an example of spectral reflection curves of two native plant flowers, and slope midpoints, which allows for the determination of the relative frequencies with which the spectral signatures could be best discriminated by a colour visual system. Figure 2 plots the frequency of the slope midpoints relative to wavelength, and the inverted $\Delta\lambda/\lambda$ function [24], which shows how hymenopteran trichromats best discriminate colour signals relative to wavelength. The insert in figure 2 shows a comparative dataset from the Northern Hemisphere [25]. The high degree of similarity between these two datasets strongly suggests that a process of parallel evolution in response to similar ecological constraints has occurred. Interestingly, both datasets reveal an increase in the frequency of slope midpoints at wavelengths longer than 600 nm (figure 2), which is a part of the spectrum that hymenopteran trichromats discriminate very poorly [24,60].

To further understand how flower colours are perceived by hymenopteran pollinators, the loci of flower spectral reflectance curves were plotted in a hexagon colour space to model pollinator perception (figure 3a). This distribution was analysed as a frequency distribution using the sectors shown in figure 3b. The main figure (figure 3) shows the frequency of flower loci in the hexagon colour space sectors, and a comparative dataset using the same analysis technique for flowers from the Northern Hemisphere [28]. The similarity between the datasets suggests that hymenopteran colour vision has influenced flower colour in both Australia and the Northern Hemisphere in a similar way.

(b) *Does variability between flowers of the same species explain why insect pollinators have behavioural plasticity for colour learning?*

To understand the relationship between variability of the colour signals provided by different flowers of the same species, and the limits of behavioural plasticity for colour learning in pollinators, we also determined the mean in colour loci separation for flowers of different plant species (figure 4). The mean value of flower variability (0.054 ± 0.045 s.d. hexagon colour units) falls in a range of colour discrimination that is consistent with the hypothesis H2, i.e. that colours of flowers from the same plant species are only reliably discriminated by bees that have experienced differential conditioning. A statistical analysis of the colour variability compared with the set threshold value of 0.04 hexagon units is significant from chance (one-sample t-test, $t_{110} = 3.276$, $p < 0.001$); showing that the degree of variability in natural flower colours is potentially an important problem that the visual system of pollinators has to overcome.

4. DISCUSSION

Colour is a major cue for how pollinators find flowers, and the colour perception of pollinators may influence which flower colours evolve more frequently. The current
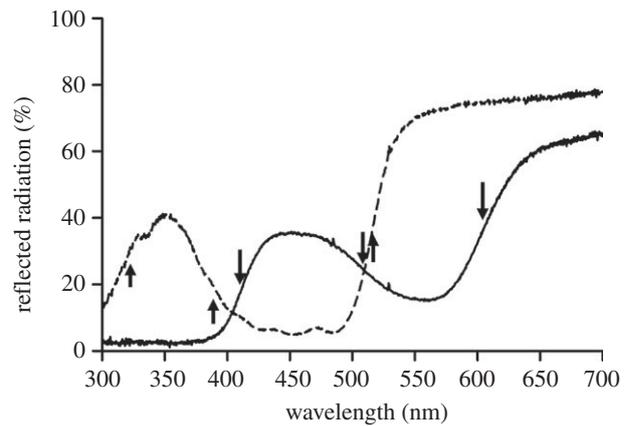
Figure 1. Example floral spectral reflections of two Australian native flowers (endemic): *Hibbertia scandens* (dashed lines, human yellow) and *Pandorea jasminoides* (solid line, human pink). The arrows show the slope midpoints [25].

study sampled a range of Australian native plant flowers and showed that the most frequent occurrence of changes in the reflectance curves fits with the regions of the electromagnetic spectrum 400 and 500 nm, where hymenopteran trichromats best discriminate spectral differences (figure 2). These data strongly suggest that hymenopteran pollinators have been a major driving force in the evolution of angiosperm flower coloration in Australia. This finding agrees with data suggesting that the majority of Australian native hymenopteran species are polylectic [30], and thus their visual capabilities can potentially influence the evolution of a wide range of flowering plants.

Another possibility that could explain the very close fit of data in figure 2 is that other potentially important pollination vectors, such as birds and/or butterflies, might also possess enhanced spectral discrimination in the 400 and 500 nm regions of the electromagnetic spectrum. The visual system of birds typically contains four spectral classes of single cones that contribute to colour discrimination [23,66,67]. While there is variability in single cone spectral sensitivity in birds [14,66,67], of the 14 avian orders tested to date, birds fall into two main groups [66]. The violet sensitive (VS) group has VS ($\lambda_{max} \sim 400-430$ nm), short wavelength sensitive (SWS; $\lambda_{max} \sim 450-480$ nm), mid wavelength sensitive (MWS $\lambda_{max} \sim 530-550$ nm) and long wavelength sensitive (LWS $\lambda_{max} \sim 600-620$ nm) spectral sensitivities considering ocular filtering, while the ultraviolet sensitive (US) group has US ($\lambda_{max} \sim 360-380$ nm), SWS, MWS and LWS spectral sensitivities. The visual behaviour of the pigeon has been well studied, and while not a major pollination vector, the $\Delta\lambda/\lambda$ function for the pigeon has been measured and is a representation of the visual capabilities of the VS group of birds. Behavioural data for wavelength discrimination by pigeons show minima at 460, 540 and 600 nm [68,69], and qualitatively similar values have been empirically measured for the hummingbird [70]. Thus, the visual system of VS birds does not correspond well with the high frequency of flower inflection points in figure 2. The budgerigar has photoreceptor peak spectral sensitivities at 365, 462, 513 and 581 nm when considering the effects of oil droplet filtering [71], and is a representative model of the US-type avian
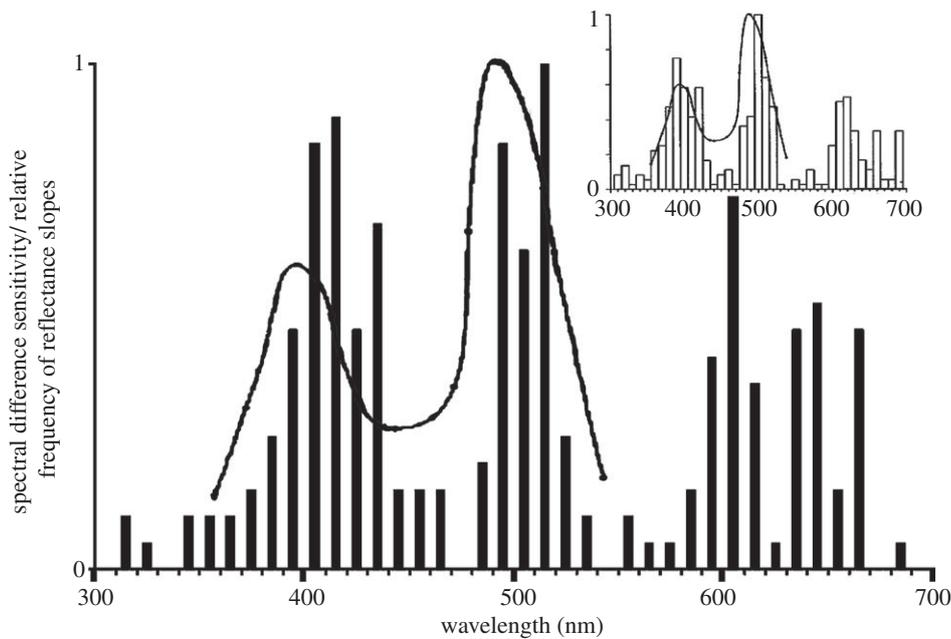
Figure 2. Relative frequency of Australian native plant flowers ($n = 111$ flowers; highest column $n = 19$ step functions) having reflectance curve slope midpoints (black bars) compared with the inverse $\Delta\lambda/\lambda$ wavelength discrimination function (solid curve) of the honeybee [24]. The insert shows data for flowers from Israel ($n = 180$ flowers; highest column $n = 36$ step functions) from a previous study that used the same methodology [25]. Australia is geologically well separated in space and time from the rest of the world and has a distinctive bee fauna [30]; the data suggest parallel evolution of many angiosperm flower signals to be best discriminated by hymenopteran trichromats.

visual system with theoretical wavelength discrimination minima based on modelling and behavioural testing at about 416, 489 and 557 nm [71]. While the 416 and 489 nm minima approximately match the spectral data in figure 2, there is no corresponding peak at around 557 nm in the flower reflectance data (figure 2), but biochemical and phylogenetic constraints should allow for these types of reflectance curves if there was sufficient evolutionary pressure [36,72], and these reflectance curves do exist at low frequencies (figure 2). This suggests that birds having a US visual system also do not match the data in figure 2.

Unlike the phylogenetically conservative spectral positions of colour photoreceptors in hymenopteran trichromats [21,22], the spectral properties of different butterflies show a large degree of diversity [73–75] and can be trichromatic, tetrachromatic or pentrachromatic [73,75]. Both molecular tuning of opsin genes [73,76] and pigment filtering [77] suggest that butterfly spectral sensitivity differences evolved relatively rapidly, leading to a large degree of diversity of colour capabilities in these insects [73]. It is thus unlikely that butterfly pollinators, when considered as a group, could explain the fit of data in figure 2, because the colour discrimination capabilities of these insects would, in some cases, predict very different flower colours. For example, the $\Delta\lambda/\lambda$ function has been measured for the butterfly genus *Papilio* and reveals three minima at approximately 430, 480 and 560 nm [78], and these minima do not fit with the data for most frequently evolving flower colours (figure 2). Another group of potentially important pollinators is flies [79]. While some flies such as *Musca* do have trichromatic spectral sensitivities close to those of hymenopteran trichromats [21,80], fly spectral sensitivities can be readily shifted with molecular manipulations to the

opsin sequence [81], suggesting different fly species do not possess colour vision that is as conserved as hymenopteran trichromats [21]. In addition, recent work on fly pollination suggests that olfaction is the main cue used by flies to discriminate between flowers, while colour is not an important cue for these pollinators [82]. It is unlikely that flies are the major driver behind the evolution of flower colours for two other main reasons: (i) as far as is currently known from behavioural experiments on flies, their colour perception is relatively rudimentary and is mediated by simple categorical colour discrimination (i.e. spectral differences are only perceived as either 'same' or 'different' to a training stimulus, depending on whether they lie inside a limited number of colour categories) [82,83]. Thus, there is currently a paucity of behavioural data on flies to support that these insects do discriminate colour information in a way that would be the major driver of flower evolution; and (ii) while there is evidence that some flies such as hoverflies do exhibit flower constancy [79], colour cues do not appear to be a factor in flower-constant behaviour in flies and these insects choose randomly between morphs varying in colour [79]. Consequently, compared with social hymenopteran pollinators [15], flies are probably less-efficient pollinators of angiosperms, although more work on this topic would be of high value.

In summary, neither the colour discrimination capabilities of birds, nor butterflies, match the close fit of flower reflectance data to hymenopteran vision at 400 and 500 nm (figure 2). In addition, fly colour discrimination capabilities and flower-constant behaviour for colour cues appear poor in comparison with hymenopteran trichromats, suggesting hymenopterans are likely to be more influential drivers of colour evolution. However, the evidence of relatively fine colour discrimination
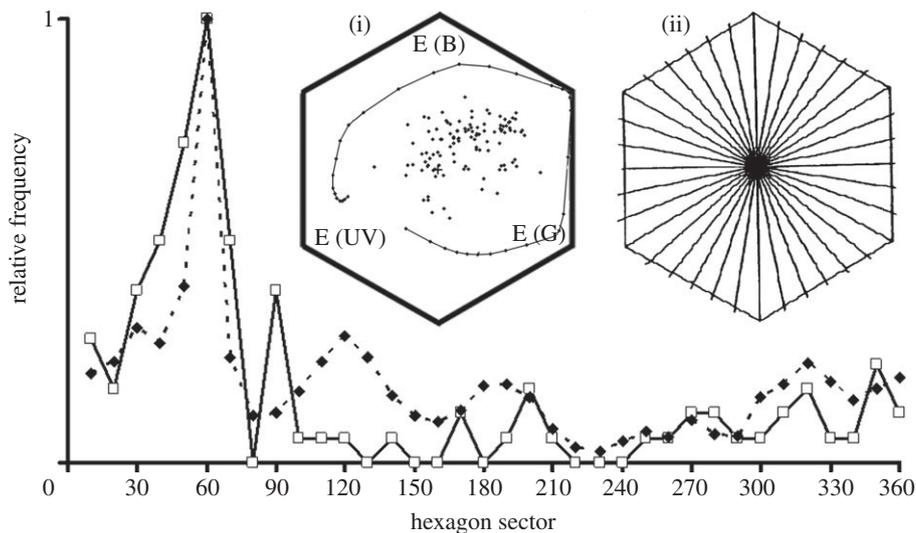
Figure 3. Relative frequencies of Australian native plant flower distributions (main figure solid line) compared with a previous study on flower evolution in the Middle East (dashed line, [28]). Data are plotted considering the visual system of hymenoteran trichromats in a hexagon colour space (see insert (i)), and the frequency with which flower colour loci were distributed in $10°$ sectors (see insert (ii); $0°$ is at the 12 : 00 angle in the colour hexagon and angles on the abscissa of the main figure read clockwise from 12 : 00) of the colour space. A similar distribution of flower colours has evolved in Australia and the Middle East, despite a very long geological separation of these study sites.
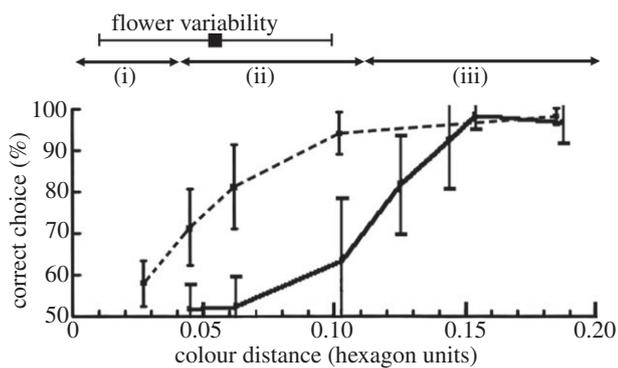

Figure 4. Colour discrimination (data from Dyer [50]) of stimuli by bumblebees (*Bombus terrestris*) considering either differential- (dashed line) or absolute- (solid line) conditioning relative to the perceptual distance in a hexagon colour space for hymenopteran colour vision (mean $\pm$ s.d.). Horizontal bars show colour distance for which: (i) colours are below threshold for either differential or absolute conditioning, (ii) colours are only reliably discriminated by bees that have experienced differential conditioning, or (iii) colours are reliably discriminated by bees even with only absolute conditioning. The variability in Australian native flowers of the same species ($n = 111$ flowers; mean $\pm$ s.d.) lies within the colour discrimination region where similar colours are only discriminated if bees have received differential conditioning (see text for statistics).

in birds and butterflies at longer wavelengths may explain the increased frequencies of some plant flowers having inflection points at these wavelengths (figure 2), although the more likely possibility is that plant material often reflects increasing amounts of radiation at wavelengths greater than about 600 nm [25,28]. Interestingly, for bird-pollinated plants, there is some evidence of both flower colours evolving spectral signals to maximize discrimination by birds [84], and/or birds evolving different spectral sensitivities to enhance discrimination of

certain flower colours [14]. This would be an interesting topic to explore considering plant flowers that are either exclusively bird, or insect-pollinated.

To interpret the current finding for Australian angiosperms in relation to a previous study that reported a link between hymenopteran colour vision and angiosperm evolution in Israel [25], it is important to consider the timeframe for geological isolation of these land regions. Australia fully separated from other major land masses around 34 Ma [31–34], but terrestrial links were tenuous before total isolation. This timeframe, and the continent's shift from high-latitude moist to mid-latitude dry climates through the Cenozoic, imposed very different pressures on the evolution of Australian plants. An alternative explanation for the similarity in the flower reflectance datasets (figures 2 and 3) is that angiosperms may have evolved particular spectral properties prior to the development of a major sea barrier, or possibly island hopped in the periods following marine separation [85], and that these early plants then had phylogentic or biochemical constraints that subsequently influenced flower evolution in Australia. Indeed by the Aptian (125–112 Myr ago), angiosperm pollen and macrofossils occur in Australia [86,87], and in roughly, coeval strata in South America, Antarctica and New Zealand [88–91]. While the pollen record for the Late Cretaceous indicate the appearance of several typical austral taxa [92] and potential sister-group relationships of these taxa to Northern Hemisphere genera [93], the plant groups extending to each hemisphere readily differentiated into discrete provincial taxa [36]. This resulted in Australia, New Zealand and Antarctica acquiring a distinctive austral flora by the end of the Mesozoic, whose genetic signature persists in the region's modern vegetation. The few fossil flowers recorded from the Cretaceous of southern Gondwana are diminutive, 'non-showy' forms with short bracts, bracteoles, tepals or petals [86,91,92]. None of these early austral fossil flowers reveals evidence of colour, which is consistent with other

evidence suggesting that early angiosperms did not have salient colour signals [94]. However, further evidence on the potential pigmentation of early angiosperms would be of high value for more fully understanding the initial stages of flower colour evolution. Plant groups with elaborate and showy flowers (e.g. *Myrtaceae, Cunoniaceae, Sapindaceae, Ericaceae, Bombacoideae, Loranthaceae, Sterculiaceae, Elaeocarpaceae, Fabaceae, Rutaceae* and *Asteraceae*) make stepwise appearances into Australasia from the early Paleogene to the early Neogene, partially spanning the terminal breakup and isolation of Eastern Gondwana [95,96]. Thus, even though angiosperms reached Australia prior to the continent's total isolation, the very large time scale suggests that flower colour evolution in Australia was probably independent to that in the Northern Hemisphere. This evidence suggests angiosperms independently evolved spectral signals and these signals were not constrained by phylogenetic contrasts of plant pigments. This conclusion is also evidenced by the data in figures 2 and 3, which shows that while certain flower colours are more frequent in nature, a wide range of flower colours can be potentially generated by plants both in the Northern Hemisphere [72] and Australia.

The evidence that angiosperms evolved spectral signals in Australia that are parallel to the evolution of flowers in the Northern Hemisphere to suit hymenopteran colour vision (figure 2) is also reflected in the similar distribution of flower colours in a colour space characteristic of bee colour perception (figure 3). Interestingly, for both study sites, there is a considerably higher frequency of flower loci in the blue to green sections (around the 60° sector) of the colour space, while loci representing pure UV and UV to blue colours are relatively rare [29,60]. One possibility for this scarcity of certain flower colours is owing to theoretical considerations that colour constancy mechanisms in bees [59,63] work poorly for UV to blue-coloured flowers owing to overlap of bee colour photoreceptors in the UV region of the spectrum [59,60], which has some empirical support from behavioural experiments [29,63].

A second important finding of the current study is that the within-species variability in flower colour is in a range that bee colour vision can only discriminate if the bees have received differential conditioning to stimuli (figure 4). This suggests that bee colour discrimination initially generalizes similar colours so that there is sufficient bandwidth to tolerate the natural variability in potentially rewarding target flowers. However, the visual system of hymenopteran insects has plasticity to learn, with differential conditioning, to make relatively fine colour discriminations and thus allow experienced individual pollinators to avoid non-rewarding mimics that are similarly coloured to rewarding model flowers if required to do so [51]. This finding helps explain how mimic (i.e. non-rewarding) flowers such as some orchids can initially gain sufficient pollinator visits to successfully reproduce despite not offering rewards, but in many cases, mimic plants remain relatively rare [97–99]. For future work, it will be of high value to understand if such non-rewarding flower species may have evolved to share similar spectral properties to rewarding flower species.

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