Red flowers differ in shades between pollination systems and across continents

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- **Background and Aims** Floral colour is a primary signal in plant-pollinator interactions. The association between red flowers and bird pollination is well known, explained by the "bee avoidance" and "bird attraction" hypotheses. Nevertheless, the relative importance of these two hypotheses has rarely been investigated on a large scale, even in terms of colour perception *per se*.

- **Methods** We collected reflectance spectra for 130 red flower species from different continents and ascertained their pollination systems. The spectra were analysed using colour vision models for bees and (three types of) birds, to estimate the colour perception by these pollinators. The differences in colour conspicuousness (chromatic and achromatic contrast, purity) and in spectral properties between pollination systems and across continents were analysed.

- **Key Results** Compared with other floral colours, red flowers are very conspicuous to birds and much less conspicuous to bees. The red flowers pollinated by bees and by birds are more conspicuous to their respective pollinators. Compared with the bird flowers in the Old World (OW), the New World (NW) ones are less conspicuous to bees and maybe more conspicuous not only to VS-birds (violet-sensitive) but also to UVS-birds (ultraviolet-sensitive). These differences can be explained by the different properties of the secondary reflectance peak (SP). SP intensity is higher in red flowers pollinated by bees than those pollinated by birds (especially the NW bird flowers). A transition from high-SP to low-SP in red flowers can induce chromatic contrast changes, with a greater effect on reducing attraction to bees than enhancing attraction to birds.

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- **Conclusions** Shades of red flowers differ between pollination systems. Moreover, red bird flowers are more specialized in the NW than in the OW. The fine evolution towards colour specialization is more likely to result in higher efficiency of bee avoidance than bird attraction.

INTRODUCTION

Colour is an important visual signal in plant-animal interactions. The colour of flowers (and sometimes floral accessories) is a primary signal in pollinator attraction (Schaefer *et al.*, 2004; Schaefer and Ruxton, 2011) and is an important dimension of pollination syndromes (Fenster *et al.*, 2004). Red floral colour has long been regarded as an important aspect of the bird pollination syndrome, combined with other characters such as tubular floral shape, copious nectar and absence of odour (Willmer, 2011). For example, Pickens (1930) found that more than 50% of the hummingbird-pollinated flowers are red or orange in the eastern United States.

Generally, signals are thought to be evolutionary results of compromise, which should be received by the intended recipients efficiently and readily while being inconspicuous to unintended recipients that have the potential to reduce the fitness of the signal senders (Endler, 2000). Two hypotheses have been proposed to explain the association between red flowers and bird pollination: bird attraction and bee avoidance (Grant, 1966; Raven, 1972). The bird attraction hypothesis is based on the fact that birds have red photoreceptors. Birds are usually tetrachromats, possessing four kinds of single cones (based on SWS1, SWS2, MWS, and LWS opsins, short-, medium, and long-wavelength-sensitive opsins; Hart and Hunt, 2007). Their red photoreceptors (LWS cones) have maximum absorbance wavelengths (λ_{max}) at 601-620 nm (Hart and Hunt, 2007) and account for most of the photoreceptors (single cones, Hart, 2001a) in the retina. Therefore, avian red photoreceptors are sensitive to the colours that humans define as red, and it is believed that red flowers are more conspicuous and attractive to birds than flowers of other colours (Grant, 1966; Chittka and Waser, 1997; Herrera *et al.*, 2008).

The bee avoidance hypothesis is based on at least three facts. First, although bees do visit flowers pollinated by birds, their pollination efficiency is generally lower than the bird

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pollinators (Castellanos *et al.*, 2003; Thomson and Wilson, 2008; Bergamo *et al.*, 2016; Krauss *et al.*, 2017). Second, their robbing behaviour may further reduce plant fitness (Irwin *et al.*, 2010; Rojas-Nossa *et al.*, 2016; but see Maloof and Inouye, 2000). Third, their ability to process red signals visually is not very good (Chittka and Wells, 2004) because they do not have red photoreceptors in their eyes. Bees have a trichromatic colour vision based on three classes of photoreceptors (UV, blue and green photoreceptors) whose maximum sensitivities are in the UV, blue and green ($\lambda_{\max} \approx 540$ nm for bumblebee *Bombus terrestris*, Skorupski *et al.*, 2007) regions respectively. This colour vision is highly conservative in Hymenopterans (Peitsch *et al.*, 1992; Dyer *et al.*, 2015), making them generally relatively weak at processing red signals chromatically. Therefore, the bird-pollinated red flowers may achieve higher fitness by remaining less attractive to the relatively inefficient visitors and flower parasites (nectar robbers).

These two hypotheses are not mutually exclusive, but their relative importance to the evolution of red flowers may not be equal. Some earlier studies implied that bird attraction could be more important (Pickens, 1930; Grant, 1966; Vickery, 1992), whereas a few more recent works support the bee avoidance hypothesis (Altshuler, 2003; Lunau *et al.*, 2011; Bergamo *et al.*, 2016). However, although behavioural experiments are essential to test these hypotheses (whether colour signal transfers to fitness difference), the estimation of colour perception *per se* may provide valuable clues. Actually, the two hypotheses may be associated with different expectations in terms of colour perception and these have seldom been tested on a large scale. If bird attraction is more important for the evolution of bird-pollinated flowers, these flowers may not necessarily be difficult for bees to detect. In contrast, if bee avoidance is more important, bird-pollinated red flowers may not necessarily be very conspicuous to birds.

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Another fact that has received less attention is that red flowers also occur in other pollination systems. Many flowers pollinated by butterflies (Johnson and Bond, 1994; Willmer, 2011) and beetles in some areas are characterized by a red colour (Dafni *et al.*, 1990). In addition, although scarce, there are indeed some plants pollinated by bees that produce red flowers (e.g., *Onosma confertum*, Chen *et al.*, in press). There may be some common associations in terms of colour perception between different pollinators and their floral colours.

More importantly, the term *red* has been defined based on the perception of humans, although it is a colour complex that may contain several colours in the pollinators' colour vision (and also in human colour vision *per se*). Based on reflectance properties, Chittka and Waser (1997) categorized human-detected red floral reflectance into three groups: flowers that only exhibit great reflectance above 600 nm; flowers that also have a secondary reflectance peak (SP) in the blue region; and flowers that have a SP below 400 nm (see Fig. 1A). Because of the spectral differences in the short wavelengths, these flower colours certainly appear different to pollinators that possess a UV receptor, such as bees (Chittka and Waser, 1997; Spaethe *et al.*, 2001; Martínez-Harms *et al.*, 2010). Lunau *et al.* (2011) discovered different UV reflectance properties between hummingbird- and bee-pollinated red flowers; and, behaviourally, bees prefer UV-reflecting more than UV-absorbing red. It seems reasonable to predict that the SP is important for pollinators that lack red photoreceptors in detecting red targets (Chittka *et al.*, 1994; Martínez-Harms *et al.*, 2010; Lunau *et al.*, 2011). However, its influence on the colour perception of pollinators possessing red photoreceptors is still not very clear.

Bird pollination occurs in many angiosperm lineages. More than one thousand bird species participate in pollination, which involves several thousand plant species in ca. 500 genera (Sekercioglu, 2006; Pauw, 2019). Bird pollinators deeply affect the evolution of

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flower traits in various aspects (Cronk and Ojeda, 2008; Krauss et al., 2017). Based on their

spectral sensitivity to short wavelength light, birds can be further divided into two colour

vision types. The UVS (ultraviolet-sensitive) type has maximum sensitivity at 355-380 nm;

whilst the VS (violet-sensitive) type is sensitive at 402-426 nm (Hart, 2001b; Ödeen *et al.*,

2009). More interestingly, there is a phylogenetic and geographic pattern associated with

avian pollinators with these two different visual systems. Studies on the amino acid sequence

of SWS1 opsins (short-wavelength-sensitive opsins) showed: sunbirds (*ca.* 132 species;

Nectariniidae), the primary avian pollinators in Asia and Africa, are UVS (λmax = 371 nm);

while hummingbirds (ca. 340 species; Trochilidae), now exclusive to the Americas, are VS

(λmax = 406 nm; Cronk and Ojeda, 2008; Ödeen and Håstad, 2010; Krauss *et al.*, 2017; but

see Herrera *et al.*, 2008). This difference in birds' colour vision has rarely considered in

previous studies on floral colour evolution (but see Burd *et al.*, 2014). Comparisons of bird-

pollinated red flowers across continents may shed light on the interaction between floral

colour and avian pollinators.

In this study, we collected floral reflectance data for red flowers native to different

continents and classified them by pollination systems. Floral colours were then analysed

using different colour vision models to estimate floral colour conspicuousness (in terms of

chromatic and achromatic contrast against the background, and purity) that may influence

pollinators' detection of flowers and their behaviour. We undertook comparative analyses and

asked the following three questions:

1) Is the red floral colour, compared with other colours, more conspicuous to birds

or/and less conspicuous to bees?

2) Is there any colour difference between bee- and bird-pollinated red flowers (hereafter

bee and bird flowers, respectively), or more generally, between flowers pollinated by animals

with different colour vision (i.e., with or without red photoreceptors, R+/-)? Considering the

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absence of red photoreceptors, we predict that red flowers pollinated by R- animals (such as

bees) tend to have high SP intensity, whereas flowers pollinated by R+ animals (e.g. birds)

may not necessarily have this characteristic.

3) For bird flowers, is there any colour difference between bird-pollinated red flowers

from the Old World (OW) and the New World (NW)? And how differently are they

perceived by birds with different colour visions? If the red colour of bird flowers has evolved

mainly to avoid bees (with relatively conservative colour vision), there may be convergent

evolution leading to similar colours that perform best in bee avoidance, regardless of how

they are perceived by birds. Whereas, if flowers have evolved to attract birds, there may be a

colour divergence between continents, optimizing conspicuousness to different birds (with

UVS- and VS-visions).

MATERIALS AND METHODS

Colour definition and data collection

Different names are used to describe various shades of human-subjective red colours, such as

scarlet, cardinal and vermilion. Despite all of them having primary reflectance at long

wavelengths (Fig. 1A), it is not easy to give a strict definition of red, as it depends on the

criteria and specific individual psychophysics. Chittka and Waser (1997) used 611 nm as the

boundary between human red and orange. However, given that bird flowers also include

some orangish colours (e.g. *Campsis radicans*), we extended this boundary to 560 nm in the

present study (also used by Reisenman and Giurfa, 2008). Therefore, the term *red* here

includes different shades of red and orange colours that mainly reflect at long wavelengths (>

560 nm). Spectral curves were collected from direct measurement ($N = 76$), the Floral

Reflectance Database (FReD, http://www.reflectance.co.uk, Arnold *et al.*, 2008; $N = 25$)

(Arnold *et al.*, 2008) and the literature ($N = 29$, detailed in Supplementary data Table S1 and

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Fig. S1). There's no personal bias in the process of data collection. We tried to collect as much data as possible to cover a relatively large range in both phylogenetic and geographical levels. These 130 red flower species belonged to 104 genera in 53 families (detailed in Supplementary data Table S1, Supplementary Data), including both monocotyledons and eudicotyledons, and are widely spread in terms of phylogeny (including Commelinids, Fabids, Malvids, Campanulids and Lamiids). In addition, their geographical distributions involve all the continents but Antarctica, which were determined based on the literature. We also collected floral reflectance data for non-red colours personally ($N = 30$) and from the FReD ($N = 282$) for comparison.

A spectrometer (USB 2000+, Ocean Optics) equipped with a UV-VIS light source (DH2000, Ocean Optics) was used to measure floral reflectance, using a pressed pellet of barium sulphate as the white standard. The fibre-optic probe was fixed at 45° to the measuring spot. At least three fresh flowers from different individual plants were used to obtain reflectance curves between 300 and 700 nm, and the average was used ultimately.

Sampled plants were assigned to a specific pollination system (i.e., pollinated by bees, hummingbirds, sunbirds, butterflies or beetles) mainly based on the available literature, involving pollinator efficiency, visitation proportion, and/or pollinator behaviour (i.e. contact with reproductive organs). When a particular group of animals contributed to > 70% of total pollen transfer, fruit/seed set, or visitations were categorized as the main pollinator(s). Visitor behaviour is also considered to determine effective pollinators and exclude visitors with no or little effect on pollination (Fenster *et al*. 2004). Very few species ($N =13$) were reported as being pollinated by two or more pollinator groups, and these were excluded from some analyses (see details on each analysis below). When the literature was limited, pollinators were determined by direct observation for 2 to 5 days of fine weather (between about 8:00 and 17:00) in the wild ($N = 14$) or botanic gardens ($N = 2$, butterfly-pollinated) for each of

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these species in successive years. In a few cases ($N = 7$) pollinators were inferred mainly

based on pollination syndromes. Although the presence of pollination syndromes has been

debated (Fenster *et al*. 2004; Ollerton *et al.*, 2009), pollination syndromes do provide clues to

infer the potential pollinator(s) when other information are not available. Pollination evidence

from congeners with very similar floral traits were considered as well. Here we considered

the main pollinators as pollinator groups while filtering out the potential noise caused by

illegal or low efficient visitors. For instance, although typical bird flowers may also be visited

by bees, their floral traits are mainly adapted to the most efficient pollinators, the birds.

Details about the plants are presented in Supplementary data Table S1.

Colour vision models

Colour vision models were used to estimate colour perception by different kinds of

pollinators. These models are established on the basis of animal anatomical and physiological

properties, combining knowledge from behavioural and/or psychophysical experiments

(Kelber and Osorio, 2010; Renoult *et al.*, 2017). Here, two widely used colour vision models

were employed: the colour hexagon model (CH model, Chittka, 1992) and the receptor noise

limited model (RNL model, Weber fraction version; Vorobyev *et al.*, 2001). The CH model is

exclusive to trichromatic Hymenoptera; it produces a two-dimensional equilateral hexagon

space in which colour perception by bees can be analysed and visualized (Fig. 1B). Colour

(chromatic) difference in the CH model is determined by the Euclidean distance between

colour loci (in CH units), and a colour locus close to the origin (we placed the green leaf

background here) is similar to the background and is difficult for bees to detect by chromatic

mechanisms (Chittka *et al.*, 1994; Spaethe *et al.*, 2001). The RNL model assumes that

receptor noise limits colour discrimination; this is applicable to both tri- and tetrachromatic

colour visions. Colour difference in the RNL model is given in "just noticeable difference"

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(JND) units. Although the RNL model was originally established for evaluating the perceptual differences of small colour differences near the detection threshold, applying it to evaluating perception of large colour differences has proved successful in research involving bees (Hempel de Ibarra *et al.*, 2001; Niggebrügge and Hempel de Ibarra, 2003) and birds (Stobbe and Schaefer, 2008; Cazetta *et al.*, 2009).

Receptor sensitivity functions and receptor noise values are the key parameters of the models. The Weber fraction, ω, was used as a substitution for the noise value (in the RNL model); as it refers to the minimum stimuli difference/change that is perceptible, and any difference smaller than the Weber fraction is unnoticeable and just perceived as noise. Receptor sensitivity functions of bumblebees (*Bombus terrestris dalmatinus*) were obtained from Skorupski *et al.* (2007), and the receptor noise values of UV, blue and green receptors (0.74, 0.67 and 0.61 respectively) from Skorupski and Chittka (2010). For birds, average receptor sensitivity functions for UVS- and VS-birds based on the model from Endler and Mielke (2005) were used. We used 0.1 as the Weber fraction for avian LWS receptors (Maier, 1992). Consequently, the corresponding Weber fraction values for UVS-birds were 0.2, 0.1414, 0.1414 and 0.1; and for VS-birds, they were 0.1414, 0.1414, 0.1414 and 0.1. Although hummingbirds' colour vision was classified as the VS-type based on the sequence of SWS1 opsins (Ödeen and Håstad, 2010), the only empirical study based on electroretinogram recordings of a hummingbird (*Sephanoides sephaniodes*) showed a λ_{max} at about 370 nm. This groups hummingbirds into the UVS-type (Herrera *et al.*, 2008; but see Ödeen and Håstad, 2010). As a reference, receptor sensitivity functions of this hummingbird were also used (Herrera *et al.*, 2008), with 0.2, 0.1414, 0.1414 and 0.1 as their corresponding Weber fraction values. Details of the models and calculations used are presented in Supplementary data Methods S1.

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For visualization, colour spaces were constructed for bees (the CH model) and birds (the tetrahedron model; Goldsmith, 1990), using the R package *pavo* (Maia *et al.*, 2013), in which colour can be presented as a point (colour locus; Fig. 1B-E). For the origin (background) of each colour space, an average of 230 leaf reflectances was used (Chittka *et al.*, 1994).

Colour conspicuousness of flowers

Colour conspicuousness is largely determined by the contrast between a target and its background, and this greatly affects the visual attractiveness to the signal receivers (Carter and Carter, 1981; Schmidt *et al.*, 2004). It includes two aspects: chromatic contrast and achromatic contrast. For bees, visual attractiveness may also be affected by purity (Lunau *et al.*, 1996; Rohde *et al.*, 2013).

Chromatic contrast describes the colour contrast that excludes brightness information. For bees, this parameter was calculated in both the CH and the RNL models; for birds, it was calculated in the RNL model only. A larger chromatic contrast value indicates a stronger flower-leaf contrast in colour, which facilitates detection.

Achromatic contrast refers to the brightness difference between a target and its background. Here, it was calculated following a previous study by Papiorek *et al.* (2015), dividing flower-leaf background contrast in green photoreceptors (for bees) or in double cones (for birds) by the Weber fractions of corresponding photoreceptors. For birds, the double cone spectral sensitivity functions of blue tit (*Cyanistes caeruleus*, Hart *et al.*, 2000), domestic chicken (*Gallus gallus*, Osorio *et al.*, 1999) and a hummingbird (*Sephanoides sephaniodes*, Herrera *et al.*, 2008) were used for UVS-birds, VS-birds and hummingbird models respectively; and we assumed their corresponding Weber fraction values are all 0.05 (Siddiqi *et al.*, 2004).

Purity refers to the saturation of colours, or how "vivid" the colours were perceived to be by animals. The effect of purity on visual attraction has been verified only in bees at present, which prefer colours with higher purity (Rohde *et al.*, 2013). This parameter was estimated in the CH model, dividing the flower-background distance by the corresponding monochromatic light-background distance (Lunau *et al.*, 1996).

Comparisons between red and other floral colours

Colour conspicuousness of red flowers ($N = 130$) and six other colour categories (namely blue, $N = 41$; pink, $N = 38$; purple, $N = 21$; violet, $N = 38$; white, $N = 86$; yellow, $N = 88$; Supplementary data Fig. S2) were compared to examine whether human red is indeed the most conspicuous colour to birds or/and the least conspicuous colour to bees (phylogenetic ANOVA, detailed below in Phylogenetic constraints).

Shades of red in different pollination systems and on different continents

Colour conspicuousness between bee flowers ($N = 10$), OW-bird flowers ($N = 37$) and NW-bird flowers ($N = 47$) were compared from the perspectives of different pollinators to explore the potential differences in red colouration between pollination systems (phylogenetic ANOVA). Given the potential difference between bird flowers within the OW, this was further divided into Asia and Africa to make comparisons between different continents (Asia, Africa, and the Americas; phylogenetic ANOVA).

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Secondary peaks in red flowers

We assumed that the difference in colour conspicuousness between flower groups largely stems from the difference in their spectral properties, especially the intensity of secondary reflectance peaks (SPs). To examine this, we calculated a ratio, k, between the height of the SP and the height of the whole spectrum, which was used to quantify the intensity of a SP and to perform the subsequent analyses.

SP Intensity pattern First, the intensity of SP in red flowers was compared between pollination systems (bee, OW- and NW-bird flowers). Then, to explore a more general association between spectral characteristics and animal colour vision properties, red flowers ($N = 110$; species whose pollinators lack clear colour vision information were excluded, e.g., certain butterflies or those flowers with a mix of different pollinator groups) were divided into two groups: those pollinated by animals equipped with red photoreceptors (R+ animals, such as birds, some beetles and butterflies) and the those by animals without such receptors (R- animals, i.e., bees, see Supplementary data Table S1 for details).. The SP intensity of these two flower groups was compared (phylogenetic ANOVA).

Influence of SP on conspicuousness To explore the effect of SP on floral conspicuousness, we used phylogenetic linear regression analyses (detailed below in Phylogenetic constraints) for each conspicuousness parameter against SP intensity.

Phylogenetic constraints

To control the potential influence of phylogeny on the evolutionary patterns of colour phenotype, we performed phylogenetic ANOVAs using the function *phylANOVA* (with 1000 simulations and Holm's method for *P* value adjustment) in the package *phytools* (Revell, 2011). For phylogenetic regression, the function *pgls* in the package *caper* was used (Orme *et al.*, 2018). Phylogenetic constraints were adjusted by estimating the maximum likelihood of Pagel's λ (Pagel, 1999). The phylogenetic tree of our sample species (Supplementary data

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Figs S1 and S2) was constructed using the *phylo.maker* function implemented in R package *V.PhyloMaker* (Jin and Qian, 2019), which uses a dated mega-tree derived from the research of Zanne *et al.* (2014) and Smith and Brown (2018) as a backbone. All the 85 families and 282 (95.3%) out of the 296 genera in our study were found in that mega-tree. For the species absent from the mega-tree, they were added using the *Scenario 3*. All statistical analyses were conducted in R 3.5.0 (R Core Team, 2018).

<div align="center">RESULTS</div>

General pattern

Of the 130 red flower species (belonging to 104 genera in 53 families) that we investigated, there were ten (7.7%, one from the NW and the others from the OW) pollinated by bees, 37 (28.5%, all native to the OW) pollinated by sunbirds (and by several other OW-birds), 47 (36.2%, all native to the NW) pollinated by hummingbirds, five (3.8%) by beetles, 18 (13.8%) by butterflies and 13 (10%) by more than one pollinator functional group (see details in Supplementary data Table S1). Our data are not seriously influenced by redundancy from the perspective of taxonomy: Most genera ($N = 90$, 86.5%) of our data contain one plant species. The biggest genera here, *Rhododendron*, has the seven species. The other genera contain no more than three species respectively. About half of the families contain one genus ($N = 28$, 52.8%). Only six families contain more than 3 genera (Fabaceae, $N = 7$; Solanaceae, $N = 7$; Bignoniaceae, $N = 5$; Gesneriaceae, $N = 5$; Lamiaceae, $N = 5$; Asteraceae, $N = 4$), which should reflect the sizes of these families. In addition, the phylogeny-informed method used in the following analyses, which considering the influence of phylogeny on analysis, can mitigate the potentially slight redundancy and imbalance to some extent.

In the bees' colour space (CH model, Fig. 1B), floral colour loci were generally clustered around the central zone, so may be perceived as colours very similar to the leaf

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background (the origin) by bees. To birds (in the tetrahedron models, Fig. 1C-E), the flower colour loci mainly clustered along the axis of the red photoreceptors. Colour loci showed a more dispersed distribution in the model spaces for UVS-birds (Fig. 1C) and hummingbirds (Fig. 1E) than VS-birds (Fig. 1D), which may reflect the inherent difference between these colour vision systems.

Comparison between red and other floral colours

Chromatically, to bees, human red floral colour appears less conspicuous on the leaf background than the other six floral colours we considered. Red flowers showed a much lower chromatic contrast (P values < 0.05 for all, Fig. 2A,C) and a much lower purity (P values < 0.05 for all, Fig. 2B). For birds (UVS-birds, VS-birds, and hummingbirds), however, red flowers showed the highest chromatic contrasts among the various floral colours (P < 0.05 for all, with the exception of yellow and purple, violet and blue on some occasions, Fig. 2D-F). Achromatically, to bees, red exhibited lower contrast than white and higher contrast than the other colours (except yellow, Fig. 2G). To birds, red had lower achromatic contrast than white and yellow usually, but showed no significant differences to other colours (Fig. 2H-J).

Colour conspicuousness of red flowers

Chromatic contrast To bees, chromatic contrast of bee-pollinated red flowers was higher than that of bird-pollinated flowers under both the CH model (P = 0.003 for both comparisons with OW- and NW-bird flowers, Fig. 3A) and the RNL model (P = 0.003 for both comparisons with OW- and NW-bird flowers, Fig. 3C). To birds (UVS-birds, VS-birds and hummingbirds), however, the NW-bird flowers exhibited higher chromatic contrast than not only the bee flowers (P = 0.012, 0.008 and 0.036, respectively, Fig. 3D-F), but also the OW-

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bird flowers (P = 0.003, 0.006, 0.003, respectively, Fig. 3D-F). The better performance of the

NW-bird flowers in UVS-birds' vision was unexpected. The difference between OW-bird

flowers and bee flowers was not significant (Fig. 3D-F).

Purity Purity was higher in bee flowers than in the OW- and NW-bird flowers (each P =

0.003), and it was similar between the two groups of bird flowers (Fig. 3B).

Achromatic contrast For any vision systems (bees', UVS-birds', VS-birds' and

hummingbirds' vision), there were no significant differences in achromatic contrast between

any floral groups (Fig. 3G-J).

Geographic pattern Further comparisons of bird flowers across the three continents (Asia,

Africa and the Americas) from the perspectives of birds (UVS-birds, VS-birds and

hummingbirds) showed that the NW-bird flowers had higher chromatic contrast than the

Asian (P = 0.006, 0.032, 0.026, respectively, respectively) and African ones (P = 0.003,

0.015, 0.009, respectively; Fig. 4D-F). There was, however, no significant difference between

Asia and Africa (Fig. 4D-F)).

Secondary reflectance peak in red flowers and its colour properties on perception

The intensity of the SP was higher in bee-pollinated red flowers than in the OW and

NW bird flowers (P = 0.022 and 0.003, respectively; Fig. 5A), and higher in OW-bird

flowers than in NW-bird flowers (P = 0.022, Fig. 5A). The OW was further divided into Asia

and Africa. Asia showed higher SPs than the Americas (P = 0.030, Fig. 5A). However, Africa

showed no difference to Asia nor to the Americas (P = 0.363 and *0.082*, respectively).

Flowers were then divided into two groups depending on whether their pollinators had a

red receptor or not. Comparative analysis showed that flowers pollinated by R- animals (bees

and *Danaus* butterflies) tended to have a higher SP, whereas those pollinated by R+ animals

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(such as beetles, some butterflies, and birds) tended to have a lower or no SP ($P = 0.005$, Fig. 5B).

Phylogenetic regression analyses revealed that SP can affect floral conspicuousness significantly. For bees, there were positive correlations between floral chromatic contrast and SP intensity in both the CH ($R^2 = 0.270$, $P < 0.001$; Fig. 6A) and the RNL models ($R^2 = 0.151$, $P < 0.001$; Fig. 6C). Similarly, a positive correlation was found with respect to purity ($R^2 = 0.305$, $P < 0.001$; Fig. 6B). For birds, chromatic contrast was inversely correlated with SP intensity ($R^2 = 0.156$, $P < 0.001$; $R^2 < 0.122$, $P < 0.001$; and $R^2 = 0.074$, $P = 0.001$ in UVS-birds', VS-birds' and hummingbirds' vision, Fig. 6D-F). In other words, a weak/absent SP reduced the colour conspicuousness of red flowers to bees, while simultaneously increasing the chromatic contrast to birds. For bees, the decreasing SP intensity reduced the chromatic contrast dramatically, from above 1 JND (the theoretical discrimination threshold) to less than this value (Fig. 6C). While, for birds, although chromatic contrast increased significantly along with the decreasing of SP intensity, the absolute values were quite high (> 10 JNDs generally).

<div align="center">DISCUSSION</div>

Dual functional role of red floral colour

We investigated 130 species that produce red flowers. Compared with the other six colour categories, red is indeed (one of, at least) the most conspicuous colour to birds, and also the least conspicuous colour to bees, chromatically. The colour stimulates red photoreceptors in avian eyes strongly, while it stimulates other receptors weakly. Based on the colour opponency mechanism (which underlies the CH and RNL models), this extreme inequality of stimulation in different receptors should translate into strong colour perception of birds. In contrast, most bees have no red photoreceptors. Only green receptors were weakly excited by

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the red signals, making bees perceive true red as green. For birds, however, the absolute

mean chromatic contrast values of all the floral colours we investigated were rather large (> 5

JNDs; except white in VS-birds' vision model, 3.52 JNDs). This implied that the relative

advantage of red colouration, although it occurs in perception estimation, may not necessarily

contribute to higher attractiveness to the pollinators in the field, as any of the colours we

tested are very likely to be detected readily (Lunau and Maier, 1995; Heystek *et al.*, 2014;

Bergamo *et al.*, 2016). To bees, however, the absolute mean value of flower-background

chromatic contrast of red flowers (1.07 JNDs) was quite small and close to the discrimination

threshold of the corresponding model, meaning that it is difficult for bees to detect red

flowers, especially in a complex leafy background (Rivest *et al.*, 2017; Telles *et al.*, 2017). In

addition, the chromatic contrast was much lower than that of other floral colours. When

flowers of other colours are available in the wild, red flowers will probably be an even more

unlikely choice for bees, but will be more profitable for birds (in a resource partitioning

scenario where bees and birds compete for nectar, Rodríguez-Gironés and Santamaría, 2004).

Achromatically, red is not the least stimulating colour to bees, suggesting the possibility

that red can be detected by bees through an achromatic mechanism (Martínez-Harms *et al.*,

2010). However, this mechanism is more sensitive to light conditions and backgrounds and

thus is a relatively unstable cue for pollinators (Kelber *et al.*, 2003). In fact, it has been

suggested that the achromatic mechanism is mainly involved in detecting motion, edges

(Lehrer *et al.*, 1990) and small targets (Giurfa *et al.*, 1997) rather than being involved in

colour choice (Backhaus, 1991; Vorobyev and Brandt, 1997). Furthermore, it is more time-

consuming for detecting colour stimuli than the chromatic mechanism (Giurfa *et al.*, 1996;

Spaethe *et al.*, 2001). Therefore, the dual functional role of red floral colour in avoiding bees

and attracting birds may be delivered mainly through the chromatic mechanism (see Schaefer

et al., 2006).

Shades of red between pollination systems and across continents

The differences in the shades of red flowers among pollination systems were subtle but significant. Generally, bee flowers, with higher chromatic contrast and purity, were more conspicuous to bees. Correspondingly, bird flowers performed better with respect to bird perception. Within bird pollination systems, the NW-bird flowers, with higher chromatic contrast than OW-bird flowers (either as a whole, or separately as bird flowers from Asia and Africa), may be more conspicuous to VS-birds. Unexpectedly, these NW-bird flowers may also be more attractive to UVS-birds (e.g., sunbirds from the OW), despite the different colour vision between UVS- and VS-birds (Ödeen and Håstad, 2010).

Although many bird-pollinated red flowers are also visited by bees (see Chittka and Waser, 1997), red flowers solely pollinated by bees are rare in nature. Only ten species were included (nine of them from the OW) in this study, despite paying special attention to them. Biochemically, it is not difficult to have an evolutionary transition from non-red (e.g., blue or purple, the typical bee flower colours) to red (Rausher, 2008; Tanaka *et al.*, 2008). Therefore, this rarity *per se* may suggest an evolutionary disadvantage for bee-pollinated flowers to be red.

The influence of a secondary peak on colour conspicuousness

The differences in shades of red mentioned above may largely stem from the different properties of SP in floral reflectance. Flowers pollinated by R- animals (e.g. bees) had higher SPs, possibly because a SP is an essential component for these animals to detect a signal through chromatic contrast and purity channels (Lunau *et al.*, 1996; Lunau *et al.*, 2011). In contrast, SPs were lower in flowers pollinated by R+ animals, e.g., birds. To birds, the SP reflectance decreases the chromatic contrast of the flower colour, possibly because the

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increased stimulation in avian SWS receptors (caused by the SP) counterbalances the

stimulation in LWS receptors (excited by the primary reflectance at long wavelengths)

through colour opponency mechanisms. A considerable proportion of red flowers that we

collected are pollinated by butterflies. However, because not all flower-visiting butterflies

have red photoreceptors, and their colour vision is not conservative enough even at the family

level (Briscoe and Chittka, 2003), the SP pattern in butterfly flowers is not clear at present,

but worth examining in the future. It is worth noting that those red flowers pollinated by

beetles in this study all had low SPs (k ranges: 0.014-0.146; means ± s.e.: 0.090 ± 0.025).

Although most beetles lack red photoreceptors, the flower-visiting taxa are some of the

exceptions (Martínez-Harms *et al.*, 2012), providing evidence to support this conclusion.

The conspicuousness difference between the two bird pollination systems (from the OW

and the NW, respectively) can also be explained by much lower SP intensity in NW-bird

flowers. Given that red flowers with low-SPs (possessing low SP intensity) perform better

than flowers with high-SPs (possessing high SP intensity) in avoiding bees (and possibly also

in attracting birds) in terms of colour perception, this characteristic represents a more

specialized floral colour phenotype in bird pollination systems. Therefore, the colours of bird

flowers are more specialized in the NW than in the OW. This also coincides with the pattern

found by Fleming and Muchhala (2008) that bird pollination systems are more specialized in

the Neotropics than the Palaeotropics. Their conclusion was based on species richness data,

while our study confirmed this pattern in a specific trait. This geographic pattern may be

explained by the difference in flower-bird interaction between the OW and the NW (with a

more specialized interaction in the NW; Zanata *et al.*, 2017), or by the difference in the

diversity (higher in the NW) and spatiotemporal predictability (higher in the Neotropics) of

floral resources between continents (Fleming and Muchhala, 2008). We assume that more

intense competition between birds and bees or more frequent nectar robbing by bees (Rojas-Nossa *et al.*, 2016) in the NW may also act as a selection force and contribute to this pattern.

The function and evolution of red floral colour

Being red endows flowers with the dual-function of avoiding bees and attracting birds; being more specialized in red (with low-SP) may result in more effective bee avoidance. There may be a fine transition from high-SP to low-SP red, towards a more specialized colour phenotype, which is likely to be biochemically derived. Anthocyanin, a common and widespread plant pigment, contributes to various floral colours from blue to red. It includes three major classes: pelargonidin-, cyanidin- and delphinidin-based anthocyanins (reviewed by Rausher, 2008), which alone (e.g. pelargonidin-based anthocyanin) or in combination (sometimes associated with carotenoids, Ng and Smith, 2016) may produce a red colour. A simple dehydroxylation process (loss-of-function mutation) enables the transformation from one anthocyanin class to another, shifting colour from blue gradually to redder colours (reviewed by Rausher, 2008). We noticed that although the NW flowers were dominated by low-SP red flowers, high-SP flowers still exist, suggesting the possibility of a gradual transition. Future studies based on a clear phylogenetic framework will be helpful to examine this shift.

Based on colour perception estimation and previous behavioural studies, we infer that better bee avoidance, rather than bird attraction, may have promoted this delicate shift. First, red flowers with high-SP are conspicuous enough to birds (chromatic contrast > 10 JNDs generally, Fig. 6D-F), the further increase in colour conspicuousness seems to be relatively unimportant. In contrast, this change could be crucial to bees, resulting in the chromatic contrast decrease from above 1 JND to less than 1 JND (Fig. 6C). This may greatly impede the target detection through the chromatic mechanism. Second, the perception difference

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between high-SPs and low-SPs to birds seems not to lead to differential behavioural responses (see Meléndez-Ackerman *et al.*, 1997; Lunau *et al.*, 2011). Some birds even respond similarly between very different colours, e.g., white and pink, in the field (Bergamo *et al.*, 2016; but see Heystek *et al.*, 2014). For bees, however, it has been shown that the colour perception difference can successfully translate into different behavioural responses (Lunau *et al.*, 2011; Chen *et al.*, in press).

To conclude, our results revealed the difference in shades of red between bee and bird flowers, and showed that such a difference may be common between flowers pollinated by R- and R+ animals. We also found a subtle but significant colour difference between red avian flowers from different continents. Unexpectedly, although differing in their colour vision, the visual selection by UVS- and VS-birds with respect to shades of red may be similar. Low-SP red is a more effective colour than high-SP red for bee avoidance, representing a more specialized colour phenotype. And red flowers are more specialized in the NW than the OW. We inferred a delicate shift from high-SP to low-SP red in bird-pollinated flowers, which was more likely to have been driven by avoiding bees than attracting birds.

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LEGENDS TO FIGURES

Figure 1 Characteristics of floral reflectance spectra, and colour loci of 130 red flower species in the study according to different colour vision models. (A) Reflectance spectra of *Onosma confertum* and *Passiflora coccinea*, as representatives of red flowers with and without a secondary peak (SP). (B) Colour loci in a colour hexagon (CH) model for bees; purple, blue and green vertices represent the maximum signals, 1, in UV, blue and green photoreceptors. (C), (D) and (E) Colour loci in the tetrahedron colour spaces for UVS-birds, VS-birds and hummingbirds; the inverted triangles in the centres refer to the origin points; purple, blue, green and red vertices represent the maximum signals (relative quantum catches), 1, in SWS1, SWS2, MWS, and LWS-receptors. Point brightness represents SP intensity of each floral spectrum, with darker points corresponding to spectra with lower SPs.

Figure 2 Comparisons of parameters of colour conspicuousness between human-subjective red (R) and the other six floral colours according to different colour vision models. The six other floral colours are blue (B), pink (Pi), purple (Pu), violet (V), white (W), yellow (Y). The numbers at the bottom of the columns in (A) are sample sizes. The receptor noise limited (RNL) model was used for both bees and birds, and the colour hexagon (CH) model was used in addition for bees. (A) and (B) Chromatic contrast and purity in bees' vision according to the CH model; (C), (D), (E) and (F) chromatic contrast in bees', UVS-birds', VS-birds' and hummingbirds' vision according to the RNL model; (G), (H), (I) and (J) achromatic contrast in bees', UVS-birds', VS-birds' and hummingbirds' vision. *phylANOVAs* (with 1000 simulations and Holm's method for *P* value adjustment) were used. Values are shown as means ± s.e. Asterisks indicate significant differences (n.s. = not significant; *, $P < 0.05$).

Figure 3 Comparisons of parameters of colour conspicuousness between red flowers pollinated by bees, OW-birds and NW-birds according to different colour vision models. The receptor noise limited (RNL) model was used for both bees and birds, and the colour hexagon

(CH) model was used in addition for bees. (A) and (B) Chromatic contrast and purity in bees' vision according to the CH model; (C), (D), (E) and (F) chromatic contrast in bees', UVS-birds', VS-birds' and hummingbirds' vision according to the RNL model; (G), (H), (I) and (J) achromatic contrast in bees', UVS-birds', VS-birds' and hummingbirds' vision. *phylANOVAs* (with 1000 simulations and Holm's method for *P* value adjustment) were used. Values are shown as means ± s.e. Different letters above the error bars indicate the corresponding significant differences at 0.05 level (n.s. = not significant). The numbers at the bottom of the columns in (A) are the sample sizes.

Figure 4 Comparisons of parameters of colour conspicuousness between bird-pollinated red flowers from Asia, Africa and the Americas according to different colour vision models. The receptor noise limited (RNL) model was used for both bees and birds, and the colour hexagon (CH) model was used in addition for bees. (A) and (B) Chromatic contrast and purity in bees' vision according to the CH model; (C), (D), (E) and (F) chromatic contrast in bees', UVS-birds', VS-birds' and hummingbirds' vision according to the RNL model; (G), (H), (I) and (J) achromatic contrast in bees', UVS-birds', VS-birds' and hummingbirds' vision. *phylANOVAs* (with 1000 simulations and Holm's method for *P* value adjustment) were used. Values are shown as means ± s.e. Different letters above the error bars indicate the corresponding significant differences (n.s. = not significant). The numbers at the bottom of the columns in (A) are sample sizes.

Figure 5 Intensity of secondary peaks in different red floral groups. (A) SP intensity of red flowers pollinated by different pollinators (bees and birds on different continents). (B) SP intensity of red flowers pollinated by animals with and without red photoreceptors (R+ and R-). Dark points show the mean values of each group. *phylANOVAs* (with 1000 simulations and Holm's method for *P* value adjustment) were used. Asterisks indicate significant differences (n.s. = not significant; *, $P < 0.05$; **, $P < 0.01$).

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Figure 6 Relationship between floral parameters and the intensity of secondary peaks. Relationship between SP intensity and chromatic contrast (A), and purity (B) in bees' vision according to the CH model; and chromatic contrast in bees', UVS-birds', VS-birds' and hummingbirds' vision according to the RNL model (C), (D), (E) and (F); and achromatic contrast in bees', UVS-birds', VS-birds' and hummingbirds' vision (G), (H), (I) and (J). Phylogenetic regressions were performed using the function *pgls* in the package *caper*. The dashed line in (C) illustrates the theoretical discrimination threshold (1 JND) in the RNL model.
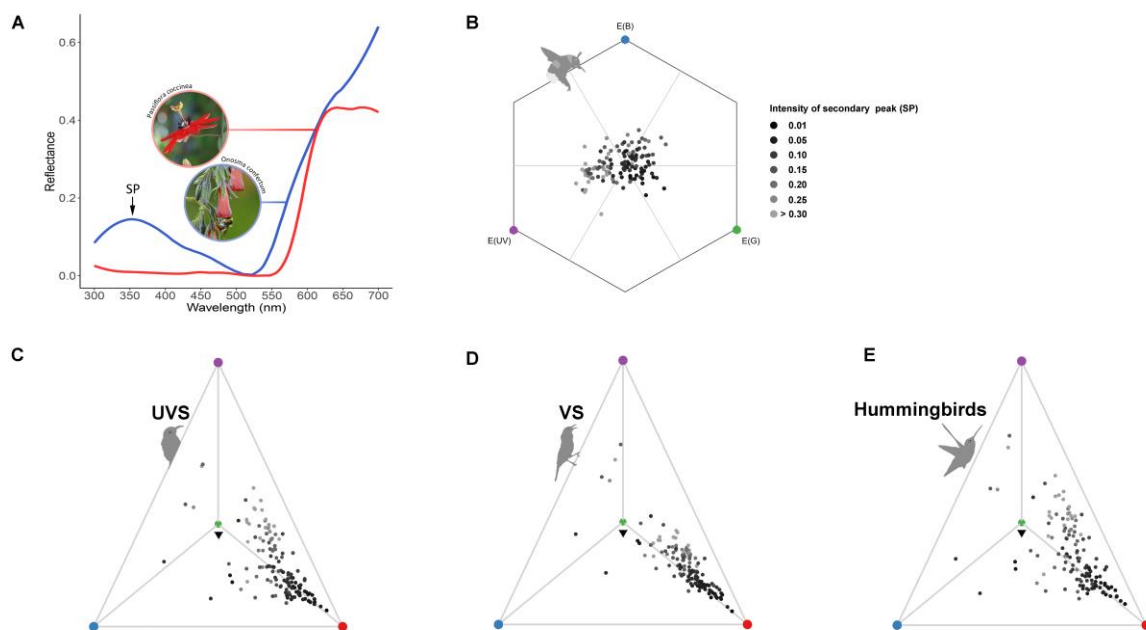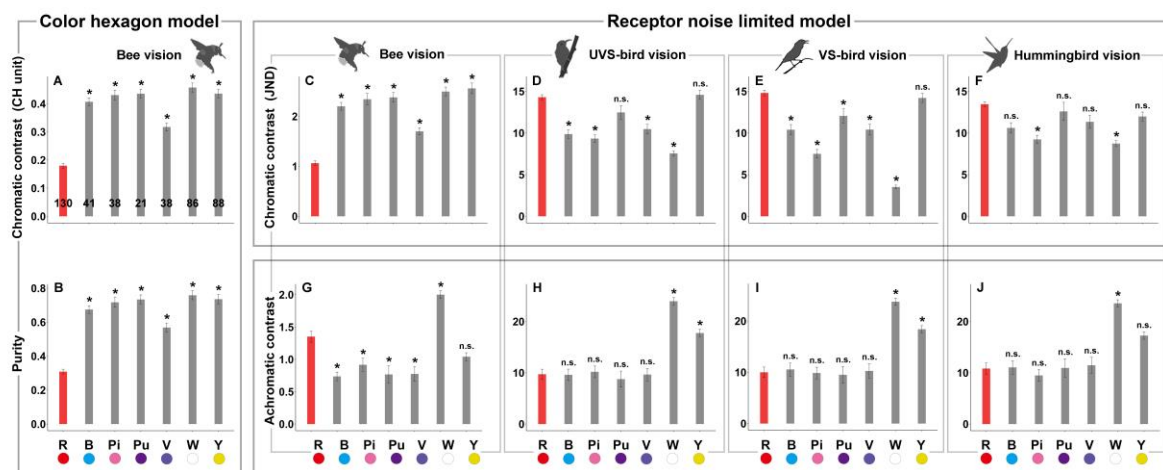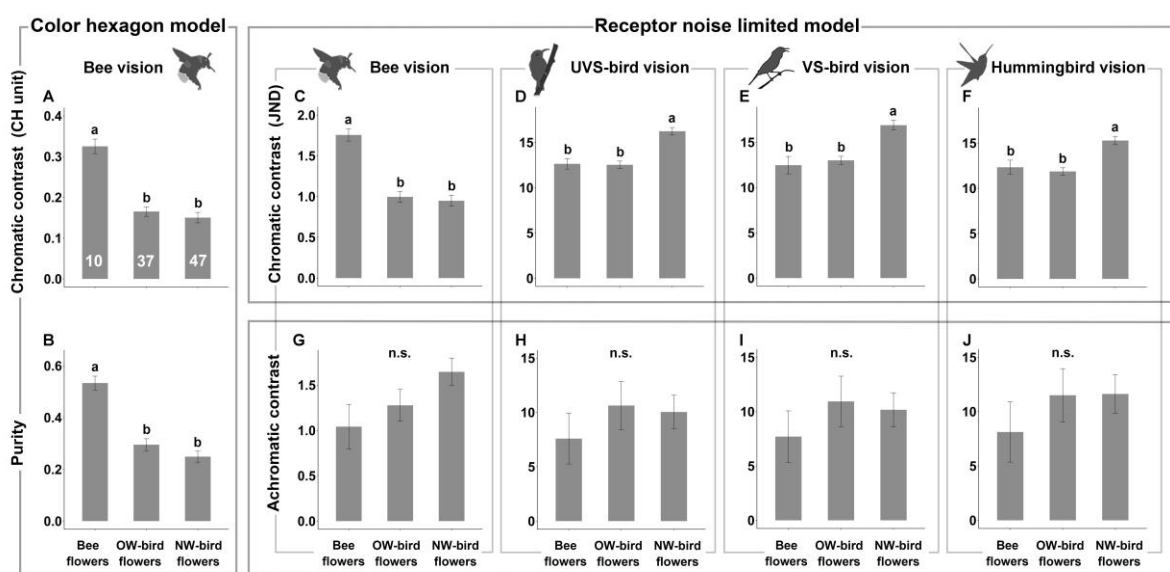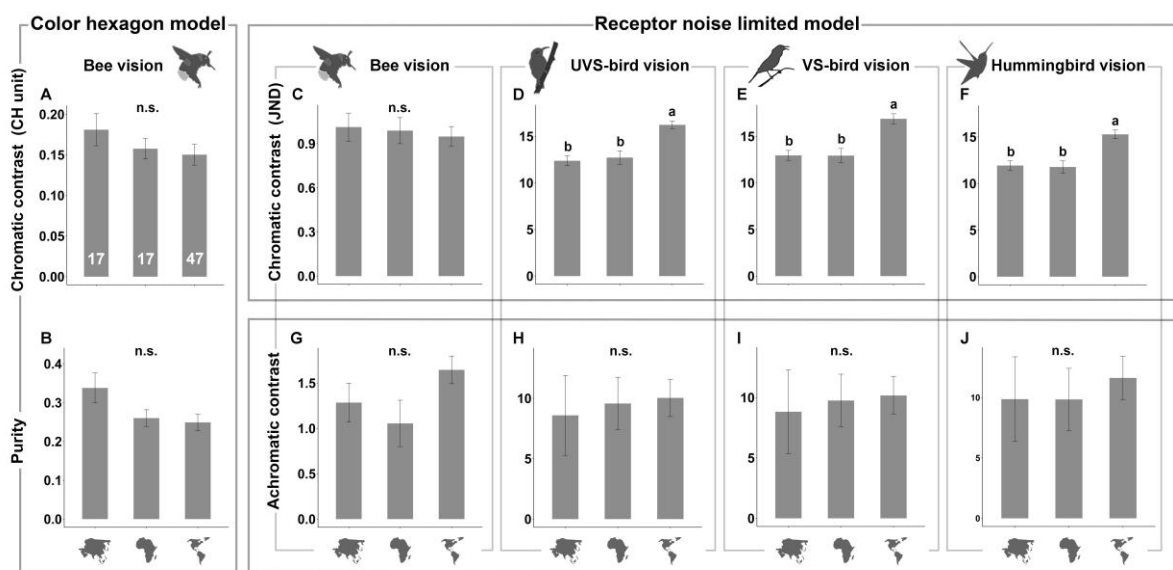
Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6