Evaluating the spectral discrimination capabilities of different pollinators and their effect on the evolution of flower colors

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Important plant pollinators like bees and birds have very different color visual systems. Previous work has attempted to relate flower syndromes to the respective visual capabilities of the most important pollinators, but has often been limited by the lack of robust means to make between-species comparisons of how flower color signals are processed. In a recent study we solved this dilemma by comparing the raw spectral signals, quantifiable by major inflection points on a wavelength scale, from different flowers whose pollinators were known from direct observation. Here we elaborate on how this method allows robust cross species comparisons that are independent of the requirement to know the complex and often inaccessible physiological data about color processing in different animals. The use of this method should thus allow for the testing of pollinator syndrome hypotheses for different animal pollinators from different regions of the world.

In recent times there has been an increased awareness that to understand how flower signals may have evolved under pollinator mediated selection, the visual ecology of floral color signals needs to be interpreted in relation to the visual capabilities of specific pollinators.[1-3] This may be a straightforward position to advance, but it remains surprising how many ecological studies evaluate flower spectral properties purely by assessment with human visual responses to color. For example, bird pollination has evolved many times in a large number of angiosperm lineages, usually from ancestors that were insect pollinated.[4-6] "Red"-colored flowers have typically been associated with bird pollination and "blue"-colored flowers with bee pollination,[7] and these associations are classically explained via optimal foraging theory and the relative efficiency of bees and birds in detecting "blue" and "red" signals respectively.[8] More recently, an extensive study of floral evolution in *Pedicularis* in the Hengduan Mountains of south-central China classified color traits by direct human observation using human defined categories like red-red, red-white, red-yellow, etc.[9]

A direct human assessment of the color of spectral signals is not necessarily an accurate way to understand how different pollinating species actually perceive floral signals.[10] The color signal that is perceived by an animal is determined by several factors, including the spectral sensitivity of photoreceptors, the number of photoreceptors and subsequent neural processing of the signals captured by the respective photoreceptors, which can incorporate complex color-opponent neural mechanisms.[11,12] The spectral sensitivity of a single type of photoreceptor (**Fig. 1**) describes the probability with which photons of various wavelengths of radiation are captured and subsequently turned into neural signals for processing in the brain. The corresponding spectral position of a photoreceptor can be specified by the wavelength at which the probability of capture is highest. For example, normal human color vision is trichromatic (i.e., has three types of cone photoreceptors) with absorbance maxima at approximately
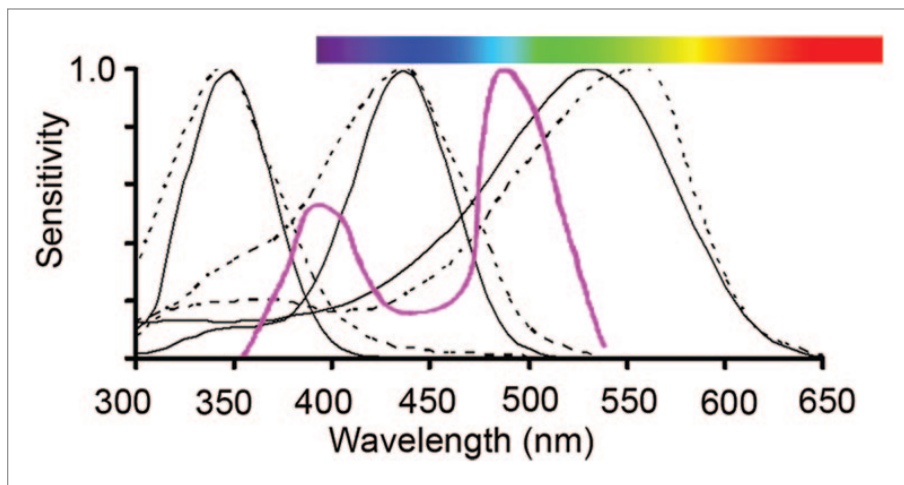
Figure 1. Important hymenopteran pollinators are trichromatic[14] and have a color visual system with UV-sensitive (maximum sensitivity about 350 nm), blue-sensitive (maximum sensitivity about 440 nm) and green-sensitive (maximum sensitivity about 540 nm) photoreceptors, while human trichromatic vision perceives longer-wavelength radiation (as indicated by the horizontal visible light spectral bar above the graph). The black line plots show sensitivity of the three photoreceptors in honeybees (dotted line)[30] and bumblebees (solid line)[41] normalized to a maximum of 1.0. Because of color-opponent processing mechanisms, the region of the spectrum where color discrimination is best is between overlapping photoreceptors, and the purple line shows an inverted Δλ/λ function that was empirically measured for the honeybee.[1,29] This allows for key critical regions of the spectrum (e.g., about 400 and 500 nm for hymenopteran pollinators) to be identified for comparison with inflection points of floral reflectance curves.[1]

420 (blue sensitive), 534 (green sensitive) and 564 (red sensitive) nm.[13] To perceive color, a visual system must have two or more types of spectrally different photoreceptors, and the perception of color is enabled by comparison of the signals from the different photoreceptor types in the brain of an animal.[12]

Studies investigating the visual capabilities of pollinating animals have shown a wide variety of color visual systems that can be used to process color information reflected from a flower.[11,14,15] For example, while important pollinators like bees are trichromatic which is in some ways similar to trichromatic color vision in humans, the spectral position of bee photoreceptors is shifted to shorter wavelengths, which means that these animals can see UV radiation but are relatively poor at discriminating long wavelength "red" radiation (**Fig. 1**). Thus, bees see colors differently to humans.[10] The color vision of other pollinating animals is even more complicated. Birds are typically tetrachromatic and have four types of photoreceptors that contribute to color vision,[11,15,16] and butterflies may have trichromatic, tetrachromatic, pentrachromatic or even higher

dimensional color vision depending upon the species.[17-21] Although there is some variability of the spectral sensitivity of the four spectral classes of photoreceptors that contribute to color discrimination in bird species,[15,16,22] of the 14 avian orders currently studied in detail bird visual spectral sensitivity falls into two main groups.[15] The violet sensitive (VS) group has VS (λ-max approx. 400–430 nm), short wavelength sensitive (SWS; λ-max approx. 450–480 nm), mid wavelength sensitive (MWS λ-max approx. 530–550 nm) and long wavelength sensitive (LWS λ-max approx. 600–620 nm) spectral sensitivities considering ocular filtering, while the UV sensitive (US) group has US (λ-max approx. 360–380 nm), SWS, MWS and LWS spectral sensitivities.

Given that the spectral input of these many photoreceptor types is subsequently processed by opponent-neural processes in the brain, which are poorly known for the vast majority of species, it is very difficult to directly interpret from physiological data how "color" signals are perceived by animal species. The field of colorimetry has tried to solve this dilemma for animal visual systems with the design of various

color spaces that can accommodate the different dimensions of color sensing, for example, tri- or tetrachromatic vision[23] and the possible use of physiological receptor noise properties to define perceived differences.[24] Unfortunately, such approaches provide very limited reliability even within key model species like the honeybee.[25] For example, color processing in both bumblebees and honeybees, which are some of the most important pollinators of plants, has been shown to be plastic and potentially dependent on top-down mediated attentional factors.[2,26,27] Indeed, even after half a century of well-funded work on human trichromatic vision and color discrimination, which is far easier to access than animal vision because human subjects will follow instructions and perform simultaneous color discrimination tasks, colorimetry has struggled to produce reliable and reproducible results based upon "just-noticeable-difference criteria" and, indeed, leading authorities like MacAdam concluded that such a goal is unattainable.[28] This means that direct comparisons of color discrimination between different species for which we know little or nothing about their complex neural processing mechanisms remains a goal that is unlikely to be resolved with colorimetric analyses any time in the near future.

How then can color evolution in flowers (or fruits, feathers, or any structures sending chromatic signals) be studied when we can't rely on human vision to interpret color differences, and colorimetry requires such detailed data about a species' color processing abilities as not to be practical or robust? A clever solution to this dilemma is to circumvent the complexities of color processing and focus on the discrimination of different wavelengths of light (radiation). These psychophysical measurements of discrimination ability are called Δλ/λ functions (**Fig. 1**), and since the experimentally determined functions measure discrimination of the whole visual process in an animal vs. a fundamental wavelength scale, it is possible to compare these data directly with the spectral "signatures" of flower spectral signals that are also measured on a wavelength scale.[1-3,29]

A potential downside to this approach is that the behavioral experiments needed

to determine Δλ/λ functions are very difficult to perform and thus, exist for only a limited number of animal species. However, in hymenopteran species the spectral distribution of photoreceptors is very conservative[14] and so it is possible to assume that the high quality Δλ/λ functions already measured for the honeybee is representative of hymenoptera trichromatic vision in general (**Fig. 1**). In addition, while avian vision does show variability between species, the presence of two main groups (VS and US) means that it is possible to select representative model species to interpret how birds may best discriminate different wavelengths of light and thus, compare data directly with flower reflectance.[2] Furthermore, these Δλ/λ functions can be predicted with reasonable reliability by comparing the regions of the spectrum where signals from different photoreceptors overlap, and where theoretical considerations of color vision suggest optimal discrimination.[29,30]

The counterpart to wavelengths of optimal discrimination by pollinators is wavelengths of floral spectral signals that have the largest effect on a pollinator's perception of color. To identify these wavelengths of greatest importance, one must remember that any individual photoreceptor type provides an essentially achromatic signal of photon catch, but the animal's brain uses opponent processes to compare the signals of different photoreceptor types to construct color perception.[11,12] Rapid changes in an object's reflectance over a short wavelength range provide the greatest information for such comparisons among photoreceptor excitation. If floral spectral signals have evolved to enhance discrimination by a pollinating animal, rapid reflectance change should occur near wavelengths of maximum discrimination, as indicated by a Δλ/λ function (**Fig. 1**).[1]

This approach has been very useful in revealing the match between floral spectral signals and hymenopteran wavelength discrimination ability in a general sample of flower colors in both the Northern Hemisphere[1] and Australia.[2] Our recent work[3] makes four important refinements of this methodology. One is that we identified the spectral location of rapid reflectance changes using an objective rule: the mathematical definition of an inflection as a point at which the second derivative is zero and the first derivative is at an extremum. This obviates the potential problems of subjective coding of data. The second advance was using independent evidence, mostly from scientific literature, of observed flower visitors for each species in our sample. Thus we were able to classify floral colors into pollination groups without assumptions based on floral morphology, human perception of colors, or other putative traits of "pollination syndromes." Third, we used a quantitative measure of the match between floral reflectance inflections and the discrimination optima identified by Δλ/λ functions for different classes of pollinators, and subjected these measures to statistical test. Finally, we used phylogenetically informed statistical tests, a necessity when dealing with multi-species data sets.

It will now be of very high value to further refine this methodology and apply it in other geographic and phylogenetic contexts. One shortcoming of previous studies[1-3] is that taxon sampling has been extremely broad and incomplete. This limits the findings to very coarse evolutionary interpretations. Demonstrations that evolutionary shifts in floral signals are tied to pollinator-mediated selection will require thorough taxonomic and geographic sampling targeted at specific lineages with known phylogenies (or simultaneous collection of sequence data for phylogenetic reconstruction). An example of an investigation with an outstanding phylogenetic and biogeographic framework is the *Pedicularis* study of Eaton et al.;[9] what it lacked was quantification of floral spectral signals and analysis with respect to pollinator visual capacities.

Similarly, investigations of floral chromatic cues in specific biogeographic contexts could be highly informative. An immediate implication of our work with Australian bird-pollinated flowers is that there should be a large-scale geographic structure to floral signals corresponding to the biogeographic distributions of major groups of nectarivorous birds. Hummingbirds (Trochilidae), a group with VS color vision, are important pollinators in the neotropics,[31] suggesting that there should be a high prevalence of flowers with spectral signals clustered close to 600 nm. This is the signature of avian pollination we found in Australia, where honeyeaters (Meliphagidae), another VS avian group, are important pollinators.[32] In contrast, a US vision group, the sunbirds (Nectariniidae), are common pollinators in many parts of Africa and Asia[31] and thus, we might expect to observe a higher proportion of flowers with inflection points clustered at shorter wavelengths. It would be interesting to see how floral color cues affect the cross-continental invasion potential of plants and how pollinators accommodate the cues of exotic plants (compare ref. 33). Another biogeographic hypothsis concerns evolution and ecological filtering along elevation gradients. It has been hypothesized that avian and hymenopteran pollinators decline with increasing altitude while fly pollination increases,[5,34-38] but whether this significantly shifts the distribution of flower spectral signals along elevation gradients remains to be tested.

Testing these and other hypotheses using a robust analytical methodology will, thus allow for comparisons between potentially different pollination syndromes around the world in a way that takes account of color discrimination capabilities of different animals, but avoids the largely unknown complexities of modeling colors in different color spaces that have no common metric to allow for meaningful comparative analyses. Targeted studies using detailed phylogenies and thorough taxon sampling will allow more definite and precise conclusions about the timing and direction of evolution of floral color signals and the role pollinators play as selective factors. The rapid expansion of analytical methods to deal with phylogenetic structure in multispecies data sets (e.g. ref. 39) will assist in uncovering the historical factors that have shaped floral color in lineages and ecological communities. Despite two centuries of pollination biology since the classic work of Sprengel,[40] the field remains open for fundamental advances in understanding how pollinator perception influences floral evolution.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Chittka L, Menzel R. The evolutionary adaptation of flower colours and the insect pollinators' colour vision. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 1992; 171:171-81; http://dx.doi.org/10.1007/BF00188925.

2. Dyer AG, Boyd-Gerny S, McLoughlin S, Rosa MGP, Simonov V, Wong BBM. Parallel evolution of angiosperm colour signals: common evolutionary pressures linked to hymenopteran vision. Proc Biol Sci 2012; 279:3606-15; PMID:22673351; http://dx.doi.org/10.1098/rspb.2012.0827.

3. Shrestha M, Dyer AG, Boyd-Gerny S, Wong BBM, Burd M. Shades of red: bird-pollinated flowers target the specific colour discrimination abilities of avian vision. New Phytol 2013; 198:301-10; PMID:23368754; http://dx.doi.org/10.1111/nph.12135.

4. Kay KM, Reeves PA, Olmstead RG, Schemske DW. Rapid speciation and the evolution of hummingbird pollination in neotropical *Costus* subgenus *Costus* (Costaceae): evidence from nrDNA ITS and ETS sequences. Am J Bot 2005; 92:1899-910; PMID:21646107; http://dx.doi.org/10.3732/ajb.92.11.1899.

5. Cronk Q, Ojeda I. Bird-pollinated flowers in an evolutionary and molecular context. J Exp Bot 2008; 59:715-27; PMID:18326865; http://dx.doi.org/10.1093/jxb/ern009.

6. Rausher MD. Evolutionary transitions In floral color. Int J Plant Sci 2008; 169:7-21; http://dx.doi.org/10.1086/523358.

7. Raven PH. Why are bird-visited flowers predominantly red? Evol 1972; 26:674; http://dx.doi.org/10.2307/2407064.

8. Rodríguez-Gironés MA, Santamaría L. Why are so many bird flowers red? PLoS Biol 2004; 2:e350; PMID:15486585; http://dx.doi.org/10.1371/journal.pbio.0020350.

9. Eaton DA, Fenster CB, Hereford J, Huang SQ, Ree RH. Floral diversity and community structure in *Pedicularis* (Orobanchaceae). Ecology 2012; 93:S182-94; http://dx.doi.org/10.1890/11-0501.1.

10. Kevan PG, Chittka L, Dyer AG. Limits to the salience of ultraviolet: lessons from colour vision in bees and birds. J Exp Biol 2001; 204:2571-80; PMID:11511673.

11. Kelber A, Vorobyev M, Osorio D. Animal colour vision—behavioural tests and physiological concepts. Biol Rev Camb Philos Soc 2003; 78:81-118; PMID:12620062; http://dx.doi.org/10.1017/S1464793102005985.

12. Dyer AG, Paulk AC, Reser DH. Colour processing in complex environments: insights from the visual system of bees. Proc Biol Sci 2011; 278:952-9; PMID:21147796; http://dx.doi.org/10.1098/rspb.2010.2412.

13. Bowmaker JK, Dartnall HJA. Visual pigments of rods and cones in a human retina. J Physiol 1980; 298:501-11; PMID:7359434.

14. Briscoe AD, Chittka L. The evolution of color vision in insects. Annu Rev Entomol 2001; 46:471-510; PMID:11112177; http://dx.doi.org/10.1146/annurev.ento.46.1.471.

15. Hart NS, Hunt DM. Avian visual pigments: characteristics, spectral tuning, and evolution. Am Nat 2007; 169(Suppl 1):S7-26; PMID:19426092; http://dx.doi.org/10.1086/510141.

16. Cuthill IC, Partridge JC, Bennett ATD, Church SC, Hart NS, Hunt S. Ultraviolet vision in birds. Adv Study Behav 2000; 29:159-214; http://dx.doi.org/10.1016/S0065-3454(08)60105-9.

17. Arikawa K, Inokuma K, Eguchi E. Pentachromatic visual system in a butterfly. Naturwissenschaften 1987; 74:297-8; http://dx.doi.org/10.1007/BF00366422.

18. Briscoe AD, Bernard GD, Szeto AS, Nagy LM, White RH. Not all butterfly eyes are created equal: rhodopsin absorption spectra, molecular identification, and localization of ultraviolet-, blue-, and green-sensitive rhodopsin-encoding mRNAs in the retina of Vanessa cardui. J Comp Neurol 2003; 458:334-49; PMID:12619069; http://dx.doi.org/10.1002/cne.10582.

19. Wakakuwa M, Stavenga DG, Kurasawa M, Arikawa K. A unique visual pigment expressed in green, red and deep-red receptors in the eye of the small white butterfly, *Pieris rapae crucivora*. J Exp Biol 2004; 207:2803-10; PMID:15235009; http://dx.doi.org/10.1242/jeb.01078.

20. Koshitaka H, Kinoshita M, Vorobyev M, Arikawa K. Tetrachromacy in a butterfly that has eight varieties of spectral receptors. Proc Biol Sci 2008; 275:947-54; PMID:18230593; http://dx.doi.org/10.1098/rspb.2007.1614.

21. Briscoe AD. Reconstructing the ancestral butterfly eye: focus on the opsins. J Exp Biol 2008; 211:1805-13; PMID:18490396; http://dx.doi.org/10.1242/jeb.013045.

22. Ödeen A, Håstad O. Pollinating birds differ in spectral sensitivity. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 2010; 196:91-6; PMID:20049459; http://dx.doi.org/10.1007/s00359-009-0474-z.

23. Goldsmith TH. Optimization, constraint, and history in the evolution of eyes. Q Rev Biol 1990; 65:281-322; PMID:2146698; http://dx.doi.org/10.1086/416840.

24. Vorobyev M, Brandt R, Peitsch D, Laughlin SB, Menzel R. Colour thresholds and receptor noise: behaviour and physiology compared. Vision Res 2001; 41:639-53; PMID:11226508; http://dx.doi.org/10.1016/S0042-6989(00)00288-1.

25. Dyer AG, Neumeyer C. Simultaneous and successive colour discrimination in the honeybee (*Apis mellifera*). J Comp Physiol A Neuroethol Sens Neural Behav Physiol 2005; 191:547-57; PMID:15871026; http://dx.doi.org/10.1007/s00359-005-0622-z.

26. Chittka L, Dyer AG, Bock F, Dornhaus A. Psychophysics: bees trade off foraging speed for accuracy. Nature 2003; 424:388; PMID:12879057; http://dx.doi.org/10.1038/424388a.

27. Avarguès-Weber A, de Brito Sanchez MG, Giurfa M, Dyer AG. Aversive reinforcement improves visual discrimination learning in free-flying honeybees. PLoS ONE 2010; 5:e15370; PMID:20976170; http://dx.doi.org/10.1371/journal.pone.0015370.

28. MacAdam DL. Color measurement theme and variations. Springer, 1985, Berlin Heidelberg New York.

29. Helversen OV. Zur spektralen Unterschiedsempfindlichkeit der Honigbiene. J Comp Physiol 1972; 80:439-72; http://dx.doi.org/10.1007/BF00696438.

30. Peitsch D, Fietz A, Hertel H, de Souza J, Ventura DF, Menzel R. The spectral input systems of hymenopteran insects and their receptor-based colour vision. J Comp Physiol A 1992; 170:23-40; PMID:1573568; http://dx.doi.org/10.1007/BF00190398.

31. Fleming TH, Muchhala N. Nectar-feeding bird and bat niches in two worlds: pantropical comparisons of vertebrate pollination systems. J Biogeogr 2008; 35:764-80; http://dx.doi.org/10.1111/j.1365-2699.2007.01833.x.

32. Paton DC, Ford HA. Pollination by birds of native plants in South Australia. Emu 1977; 77:73-85; http://dx.doi.org/10.1071/MU9770073.

33. Geerts S, Pauw A. African sunbirds hover to pollinate an invasive hummingbird-pollinated plant. Oikos 2009; 118:573-9.

34. Mani MS. Introduction to high altitude entomology: insect life above timberline in the north-western Himalayas. Methuen, London. 1962, p. 302.

35. Kevan PG. Floral colours in the High Arctic with reference to insect flower relations and pollination. Can J Bot 1972; 50:2289-316; http://dx.doi.org/10.1139/b72-298.

36. Pojar J. Reproductive dynamics of four plant communities of southwestern British Columbia. Can J Bot 1974; 52:1819-34; http://dx.doi.org/10.1139/b74-234.

37. Primack R. Insect pollination in the New Zealand mountain flora. NZ J Bot 1983; 21:317-33; http://dx.doi.org/10.1080/0028825X.1983.10428561.

38. Kearns CA. Anthophilous fly distribution across an elevation gradient. Am Midl Nat 1992; 127:172-82; http://dx.doi.org/10.2307/2426332.

39. Paradis E. Analysis of Phylogenetics and Evolution with R, 2ⁿᵈ ed. Springer, Dordrecht, 2012.

40. Lloyd DG, Barrett SCH. Floral Biology: *Studies on Floral Evolution in Animal-Pollinated Plants*. Chapman & Hall, New York, 1996.

41. Skorupski P, Döring TF, Chittka L. Photoreceptor spectral sensitivity in island and mainland populations of the bumblebee, *Bombus terrestris*. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 2007; 193:485-94; PMID:17333207; http://dx.doi.org/10.1007/s00359-006-0206-6.