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RESEARCH ARTICLE

Limited variation in visual sensitivity among bowerbird species suggests that there is no link between spectral tuning and variation in display colouration

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SUMMARY

Variation in visual spectral tuning has evolved in concert with signal colour in some taxa, but there is limited evidence of this pattern in birds. To further investigate this possibility, we compared spectral sensitivity among bowerbird species that occupy different visual habitats and are highly diverged in plumage and decoration colour displays, which are important in mate choice and possibly reproductive isolation. Microspectrophotometry of violet-, short-, medium- and long-wavelength-sensitive cones revealed no significant interspecific variation in visual pigment peak spectral absorbance values that ranged between 404–410, 454, 503–511 and 558–568 nm, respectively. Mean cut-off wavelength values for C-, Y-, R- and P-type coloured oil droplets were 418–441, 508–523, 558–573 and 412–503 nm, respectively, with values at longer wavelengths in ventral compared with dorsal retina cones. Low ocular media mid-wavelength transmission values (340–352 nm) suggest that bowerbirds may represent a transitional stage in the evolution from the ancestral violet-sensitive- to the derived ultraviolet-sensitive-type short-wavelength-sensitive-1-based visual system found in younger passerine lineages. Sequence data obtained for rod opsin and four cone opsin genes were identical at key tuning sites, except for an interspecific leucine-52-alanine polymorphism in the short-wavelength-sensitive 2 opsin. There was no obvious relationship between relative proportions of cone classes and either visual habitat or display colour. Overall, we detected little interspecific variation in bowerbird spectral sensitivity and no association between sensitivity and display diversity, which is consistent with the general trend among avian taxa.

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INTRODUCTION

Darwin (Darwin, 1871) and Wallace (Wallace, 1878) were keenly interested in the diversity of animal colouration and they recognized that colour traits were often a form of communication. Since then, the study of colour communication has become an active area of research in evolutionary biology. Some of the most important advances in our understanding of the evolution of colour communication have come from studies of signal design, i.e. investigations of why particular colour characteristics arise. One of the most significant recent developments has been the accumulation of evidence demonstrating a link between spectral tuning of visual systems and signal colouration. For example, studies on closely related fishes attribute divergence of male colour to selection for efficient signaling that is largely based on variation in tuning (e.g. Boughman, 2001; Carleton et al., 2005; Seehausen et al., 2008; Fuller and Noa, 2010), as suggested by the 'sensory drive' hypothesis (e.g. Endler, 1992; Endler and Basolo, 1998). Variation in tuning among fishes is well documented and appears to be driven mainly by the spectral distribution of habitat light and/or sensory specializations for visually demanding behaviors such as foraging (e.g. Levine and MacNichol, 1979; Lythgoe, 1979; Carleton, 2009). Conversely, it has also been suggested in fishes (Sabbah et al., 2010) butterflies (Frentiu and Briscoe, 2008; Yuan et al., 2010) and birds (Odeen et al., 2011) that signals may drive tuning. Additionally, under both scenarios, tuning has been related to differences in mate preferences and speciation.

Although birds have been an important focus of colour communication research, most comparative studies of signal design have focused more on the importance of variations in visual environment, e.g. colour contrast with signaling background and illuminant spectra, and less on variations in visual system performance (e.g. Endler et al., 2005; Doucet et al., 2007; Anciaes and Prum, 2008; Stoddard and Prum, 2008). This is mainly because the spectral characteristics of the photoreceptors of most terrestrial bird species that have been examined are similar. However, given that the spectral sensitivities of only ~30 out of an estimated 10,000 bird species have been studied in detail, a number of authors have suggested that spectral tuning in birds could be more complex and variable than currently realized (e.g. Carvalho et al., 2007; Hart and Hunt, 2007; Beason and Loew, 2008; Bowmaker, 2008; Frentiu and Briscoe, 2008; Yokoyama, 2008; Hunt et al., 2009; Renoult et al., 2010).

Colour vision in birds is based on the comparison of signals from four types of single cone photoreceptor that each contains a spectrally distinct class of light-absorbing visual pigment [for a detailed review of the avian visual system, see Hart (Hart, 2001b)]. All birds studied to date have been shown to utilize the vitamin A_1-derived visual pigment chromophore retinal; thus, visual pigment

spectral sensitivity is determined solely by the amino acid sequence of the opsin protein with which the chromophore is conjugated. The visual pigments in the four single cone types are formed by opsins that belong to the short-wavelength-sensitive 1 (SWS1), short-wavelength-sensitive 2 (SWS2), rhodopsin-like (Rh2) and medium-/long-wavelength-sensitive (M/LWS) classes and have wavelengths of maximum absorbance (λ_{max}) of ~355–426, 427–463, 499–506 and 543–571 nm, respectively. The cones containing an SWS1-based visual pigment are referred to as ultraviolet- (UVS) or violet-sensitive (VS) depending on the spectral location of the pigment λ_{max} (UVS λ_{max}≈355–373 nm; VS λ_{max}≈402–426 nm) and show the largest interspecific variation in cone type λ_{max}. The cones containing the SWS2, Rh2 and M/LWS-based visual pigments are referred to as short- (SWS), medium- (MWS) or long-wavelength sensitive (LWS), respectively.

The SWS, MWS and LWS single cones also contain pigmented or 'coloured' oil droplets (C-, Y- and R-type, respectively) that have type-specific spectral transmittance properties. Located in the inner segment of the cone, coloured oil droplets filter short wavelengths from reaching the visual pigment in the outer segment, and this has a pronounced affect on cone sensitivity. Specifically, light filtering by coloured oil droplets reduces the spectral bandwidth of the cone and shifts the peak sensitivity of the cone to a wavelength that can be much longer than the λ_{max} of the visual pigment it contains. As a consequence of this spectral tuning, there is less overlap in spectral sensitivity between the different cone types and this significantly enhances colour discrimination ability (Govardovskii, 1983; Dyer, 1999; Vorobyev, 2003). The SWS1 cones also possess an oil droplet (T-type); however, it is transparent from at least 300–800 nm and does not significantly affect spectral sensitivity. Instead, the short-wavelength limit to vision in birds is determined by the spectral absorbance of the SWS1 visual pigment and the spectral transmittance of the ocular media (lens, cornea, etc.).

Birds also possess three other classes of photoreceptor. Double cones are made of two paired cones (a primary and an accessory member) that are thought to mainly function in achromatic tasks such as motion detection (Campenhausen and Kirschfeld, 1998; Osorio and Vorobyev, 2005). Each member of the double cone typically contains the LWS visual pigment and there are greenish-yellow (P-type) droplets in the principal member and occasionally in the accessory member (A-type). Lastly, rod photoreceptors function in photon-limited conditions (e.g. at night), have no oil droplet and contain an MWS visual pigment with a λ_{max} similar to that of the MWS cones (but based on an Rh1 opsin protein).

The measurement of avian visual pigments and oil droplets using direct methods (e.g. microspectrophotometry) are rather painstaking and require access to live animals. Consequently, a number of studies have used indirect (molecular genetic) methods to infer spectral sensitivity based on opsin amino acid sequence. SWS1 visual pigment sensitivity has attracted particular attention because it is relatively highly variable among birds (e.g. Odeen and Hastad, 2003; Odeen et al., 2009; Odeen and Hastad, 2009; Capuska et al., 2011) and there has been interest in the potential adaptive significance of enhanced UV sensitivity (e.g. Bennett and Cuthill, 1994; Kevan et al., 2001; Hausmann et al., 2003; Hastad et al., 2005; Schaefer et al., 2007; Stevens and Cuthill, 2007). For example, multiple shifts of SWS1 sensitivity corresponding to variation in plumage UV reflectance among fairy wrens may reflect tuning for communication (Odeen et al., 2011). However, the spectral domain of the SWS1 visual pigment covers only a fraction of the total avian visible spectrum and the corresponding interspecific diversity of signal colouration. Also, other potentially important tuning parameters of the eye, such as light filtering by ocular media and differential distribution of cone photoreceptors, have received even less attention than photoreceptor spectral sensitivity, despite possible evidence of widespread tuning, e.g. interspecific variation in cone distribution that corresponds with species ecological differences (e.g. Goldsmith et al., 1984; Partridge, 1989; Hart, 2001a).

Considering, then, that information about bird visual systems is relatively limited, more investigation of spectral tuning is warranted before dismissing its significance as either a driving force in avian signal evolution or a response to signal divergence. To adequately address the potential importance of tuning requires close examination of the multiple visual parameters that contribute to interspecific variation in sensitivity. The best approach is to compare sensitivity among species that: (1) occupy different light habitats that might tune their visual sensitivity differently, (2) experience strong selection on colour communication and (3) are highly differentiated in signal colour. This approach has the advantage of potentially revealing whether changes in the visual system can occur among a set of related species showing distinctly different colour preferences (e.g. Loew et al., 2002; Raine et al., 2006; Seehausen et al., 2008).

The avian family Ptilonorhynchidae, bowerbirds, provides a compelling candidate for investigating the possibility of a link between spectral tuning and signal design in birds. Most bowerbird species have non-resource-based mating systems and extraordinarily complex male colour displays that are highly differentiated among species as a result of intense sexual selection. These displays include ornate plumage and colourful objects, commonly referred to as decorations, that males gather from the environment and arrange around the bower mating structure [for display descriptions, see Frith et al. (Frith et al., 2004)]. Bowerbirds have been an important model for studying mate choice and display trait evolution, with much of the research focused on their colour displays (e.g. Darwin, 1871; Gilliard, 1969; Borgia, 1985; Diamond, 1987; Borgia and Collis, 1990; Lenz, 1994; Patricelli et al., 2002; Doucet and Montgomerie, 2003; Coleman et al., 2004; Frith et al., 2004; Madden et al., 2004; Endler et al., 2005; Robson et al., 2005; Borgia, 2006; Endler and Day, 2006; Borgia et al., 2007; Borgia, 2008). Detailed empirical studies of multiple species have revealed strong selection on display colour that is based on female mate choice (e.g. Borgia, 1985; Borgia and Mueller, 1992; Madden, 2003; Coleman et al., 2004) and demonstrate species-distinct colour preferences and aversions (e.g. Borgia, 1985; Borgia et al., 1987; Diamond, 1987; Diamond, 1988; Borgia, 1995a; Borgia, 1995b; Uy and Borgia, 2000; Madden, 2003; Endler and Day, 2006). There are also large differences in visual habitats among species, i.e. from dim-lit closed canopy rainforest to sunny and open scrublands [for habitat descriptions, see Frith et al. (Frith et al., 2004)], which suggests the potential for differential visual tuning. Additionally, strong differences in colour preference between bowerbird species may drive the development of behavioral reproductive isolation (Uy and Borgia, 2000). Thus, understanding the potential role of spectral tuning in display diversity might also provide insight into bowerbird speciation.

Multiple studies have investigated the evolution of signal design in bowerbirds, yet the causes of species colour divergence are not well understood. Endler et al. (Endler et al., 2005) analyzed plumage and decoration reflectance spectra and argued that especially large differences in plumage colour between sympatric bowerbirds compared with allopatric pairs suggest a process of 'reinforcement' for species recognition (Dobzhansky, 1937). However, the accompanying evidence required to demonstrate this process, such as intraspecific character displacement from outside to within the sympatric zone, is lacking (Borgia et al., 2007; Endler, 2007). Two

other studies investigated whether decoration colour preference might be just a side effect of an adaptive preference for certain colour foods, as suggested by the sensory bias hypothesis (Ryan et al., 1990; Endler and Basolo, 1998; Ryan, 1998; Rodd et al., 2002; Smith et al., 2004). One study posited an overlap of food and decoration colour preferences across multiple species (Madden and Tanner, 2003). However, this study has been criticized by Borgia and Keagy (Borgia and Keagy, 2006) because it used coloured grapes (a popular food item for bowerbirds, but never used as a decoration) as both food and display objects, making it difficult to interpret their experiment. In contrast, Borgia and Keagy (Borgia and Keagy, 2006) used distinct food (cereal) and decoration (plastic) objects in a study of satin bowerbirds (*Ptilonorhynchus violaceus*) and showed an inverse relationship between food and display colour preference, indicating that different preferences were operating in each context. There has been only one study that has examined an aspect of visual sensitivity among bowerbirds to investigate spectral tuning as a potential cause of species display colour differences; Zwiers (Zwiers, 2009) compared SWS1 opsin sequence among 15 of the total 20 bowerbird species and found no evidence of interspecific variation in short-wavelength sensitivity based on this particular tuning mechanism. However, given that many bowerbird species use display colours that have prominent spectral reflectance characteristics, such as peak reflectance, in regions of the spectrum other than those covered by the SWS1 visual pigment (e.g. satin bowerbird: 'blue'; regent bowerbird: 'yellow'; spotted bowerbird: 'red'), a more complete investigation of bowerbird visual systems that covers their entire colour spectrum and examines multiple tuning parameters may reveal important insights into the evolution of their extraordinary display diversity.

In this study we characterized and compared bowerbird visual systems to assess spectral tuning among species that occupy a wide range of visual habitats and have differently coloured plumage and decoration displays. We studied a phylogenetically diverse sample of 12 polygynous species and one monogamous species (green catbird), which was used to ascertain the likely ancestral state of the bowerbird visual system (Kusmierski et al., 1993). All 13 species were also included in Zwiers' (Zwiers, 2009) study of bowerbird SWS1 opsin sequence variation, and include representatives of all eight currently recognized bowerbird genera and representative species from all major habitat types (Kusmierski et al., 1993; Frith et al., 2004). We sequenced SWS2, Rh2 and M/LWS opsin coding sequences for 13 species and in a subset of six species (including five polygynous bower-building species and the monogamous catbird) we quantified relative cone proportions and their distribution across the retina, measured ocular media transmission spectra and used microspectrophotometry to directly measure the spectral absorption characteristics of retinal photoreceptors, including coloured oil droplets and visual pigments. We also sequenced the Rh1 opsin in these six species to more completely characterize their full opsin complement. The data collected in this study represent the most extensive comparison of visual sensitivity within an avian family that has been performed to date, which substantially increases the number of bird species for which opsin sequence data are available for SWS2-, Rh2-, M/LWS- and Rh1-based visual pigments. These data allows us to better understand the colour preferences of bowerbirds, assess whether tuning has occurred and may be related to bowerbird display divergence, and better inform our general understanding about avian visual systems by providing detailed information about a number of visual parameters that may contribute to interspecific variation in sensitivity.

MATERIALS AND METHODS

Species studied

Blood samples collected previously from 12 bowerbird species were available to use for opsin sequence analysis (Table 1). Ocular tissue samples were collected specifically for this study from six species that were chosen to represent a variety of display colours and visual habitats (see Frith et al., 2004) and to include representatives for each of the three main phylogenetic groups within the bowerbird family: the ancestral (and monogamous) catbirds, the avenue-style bower-building clade and the maypole-style bower-building clade (Kusmierski et al., 1993) (Table 1).

Collection

Birds were captured by cage trap or mist net in New South Wales or Queensland, Australia, during November and December 2008. Collecting large numbers of bowerbirds is discouraged by wildlife

Table 1. Species, habitat, predominant display colours and data types collected

Species	No. of birds, sex	Habitat type	Clade	Prominent colours		MSP	Ocular media	cDNA	gDNA
				Plumage	Decoration				
Green catbird (*Ailuroedus crassirostris*)	1M	RF	Ancestral	Green	None	✓	✓	✓	
Tooth-billed bowerbird (*Scenopooetes dentirostris*)	1M	RF	Maypole	Olive brown	Green (leaves)	✓		✓	✓
Golden bowerbird (*Prionodura newtoniana*)	1M	RF, FE	Maypole	Yellow	Green				✓
Streaked bowerbird (*Amblyornis subalaris*)	1M	MF	Maypole	Orange/red	Blue, purple, reds				✓
Macgregor bowerbird (*Amblyornis macgregoriae*)	1M	MF	Maypole	Orange/red	Red, orange, yellow				✓
Archbold bowerbird (*Archboldia papuensis*)	1M	MF	Maypole	Orange/yellow	Blue, green				✓
Vogelkop bowerbird (*Amblyornis inornatus*)	1M	CF	Maypole	Olive brown	Blue, orange, red				✓
Regent bowerbird (*Sericulus chrysocephalus*)	2F	RF	Avenue	Yellow, black	Blue, yellow	✓	✓	✓	✓
Satin bowerbird (*Ptilonorhynchus violaceus*)	1M, 1F	RF, FE	Avenue	Blue/black	Blue, yellow	✓	✓	✓	✓
Fawn-breasted bowerbird (*Chlamydera cerviniventris*)	1M	OW, FE	Avenue	Cinnamon	Green				✓
Great bowerbird (*Chlamydera nuchalis*)	2M	OW	Avenue	Pink/lilac	Green, red, blue	✓	✓	✓	✓
Spotted bowerbird (*Chlamydera maculata*)	1M	OW	Avenue	Pink/lilac	Green, yellow, red, blue	✓	✓	✓	✓
Western bowerbird (*Chlamydera guttata*)	1M	OW	Avenue	Pink/lilac	Green, red, blue				✓

M, male; F, female.

Habitat types include rain forest (RF), forest edge (FE), open woodland and scrubland (OW), montane forest (MF) and cloud forest (CF).

There are three main clades of bowerbirds: avenue bower builders, maypole bower builders, and the monogamous and ancestral catbirds, which do not clear a court, or decorate or build a bower.

Plumage colours are constant across species, but there is considerable geographic variation in decoration colours used by some species, e.g. Vogelkop bowerbirds.

Microspectrophotometry (MSP) data may include oil droplet absorptance and visual pigment absorbance.

authorities; consequently, collection was limited to a maximum of two individuals per species. A previous study of a passerine species found no sex-related differences in visual sensitivity (Hart et al., 1998), thus we collected either sex as available. Following collection, birds were transported to the University of Queensland in Brisbane where they were housed individually in large, naturally illuminated aviaries for up to 5 days (most birds less than 3 days) and provided with ample water and food, including insects, fruits and bread. Immediately prior to enucleation for microspectrophotometry, birds were held in complete darkness for at least 1 h and then humanely euthanized *via* overdose of barbiturate anaesthetic, followed by cervical dislocation. The left eye of each bird was used for microspectrophotometry and the right eye was used for measuring the spectral transmittance of the ocular media and the relative abundance of the different types of cone photoreceptor. Experimental procedures were approved by the University of Queensland Animal Ethics Committee, the Director General of the New South Wales Department of Primary Industries Animal Welfare and Ethics Committee, and the University of Maryland Institutional Animal Care and Use Committee. Research permits were granted by the National Parks and Wildlife Services of New South Wales and Queensland.

Opsin sequence analysis

Extraction of gDNA from blood and total RNA from retinal tissue was performed using Qiagen DNeasy and RNeasy kits (Valencia, CA, USA), respectively. gDNA was extracted from blood samples of one individual from each of 12 species (Table 1) and total RNA was extracted from one individual for each of the six species from which ocular tissue was collected. Total RNA was reverse transcribed to cDNA using a polyT primer and Superscript III RT polymerase (Invitrogen, Carlsbad, CA, USA).

Degenerate PCR primers were developed for each opsin using Primer 3 (Rozen and Skaletsky, 2000) based on consensus sequences of *Serinus canaria* [GenBank accession numbers AJ277923 (SWS2), AJ277924 (MWS), AJ277925 (LWS), AJ277926 (RH1)] and *Taeniopygia guttata* [GenBank accession numbers AF222332 (SWS2), AF222330 (MWS), AF222333 (LWS), AF222329 (RH1)] (see supplementary material Table S1 for primer sequences). Isolated opsin sequences from satin and great bowerbirds were then used to design bowerbird-specific primers to amplify contiguous opsin cDNA sequences that span all seven transmembrane domains and include sites where certain amino acid substitutions have been demonstrated to change pigment spectral sensitivity in previous studies (e.g. for SWS2, 46, 49, 52, 91, 93, 94, 116, 122, 164, 207, 261, 269 and 292; for Rh2, 122, 222 and 295; and for M/LWS, 164, 181, 261, 269 and 292) (see Yokoyama, 2002; Takahashi and Ebrey, 2003; Hunt et al., 2009). Amino acid position numbering for opsins corresponds to bovine Rh1 amino acid positions (Palczewski et al., 2000). The same primers and others were also used in an attempt to sequence exons of opsin gDNA for 12 species.

PCR was performed on an Eppendorf thermocycler (Hamburg, Germany) using either *Taq* (Invitrogen) or Dynazyme (NEB, Thermo Fisher Scientific, Waltham, MA, USA) polymerase and following manufacturer protocols, optimized when necessary. PCR products were purified using QIAquick kits (Qiagen) and then cycle-sequenced using primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA). Sanger sequencing was performed on either an ABI 3730xl or a 3100 DNA Analyzer (Applied Biosystems). All gene fragments were sequenced in both directions.

Chromatographs were imported into Sequencher version 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA) to align sequence data and translate from nucleotide to amino acid sequence. Amino acid sequences were then compared between species and conspecific individuals to identify residues at functionally important loci (Yokoyama, 2000; Hunt et al., 2009). Bio-edit was also used for sequence aligning, editing and analysis (Hall, 1999).

Microspectrophotometry of visual pigments and oil droplets

Preparation of retinal tissue for microspectrophotometric analysis has been described in detail elsewhere (Hart, 2002; Hart, 2004; Hart et al., 2011). Briefly, dark-adapted eyes were dissected under infrared illumination with the aid of an image converter in cold (4°C) phosphate-buffered saline (PBS) (340 mOsmol kg^{-1}, pH 7.2; Oxoid, Thermo Fisher Scientific Australia Pty Ltd, Thebarton, SA, Australia). Small (1–2 mm^{-2}) pieces of retina were mounted in a drop of PBS solution containing 8–10% dextran (MW 282,000; Sigma D-7265) when measuring primarily the spectral absorbance of visual pigments in the outer segment (but also the associated oil droplets to establish visual pigment–oil droplet pairings). The spectral transmittances of oil droplets that are reported in the Results were measured from pieces of retina mounted in pure glycerol; the refractive index of glycerol is more closely matched to that of the oil droplets and results in less scattering of the measuring beam and, therefore, superior spectra. Oil droplets were measured in retinal samples taken from both the dorsal and the ventral retinal periphery in an attempt to identify systematic variations in their spectral transmittance characteristics across the retina.

Transverse spectra (330–800 nm) of rod and cone outer segments and cone oil droplets were made using a single-beam, wavelength-scanning microspectrophotometer (Hart et al., 2011). A sample scan was made by aligning the measuring beam (typical dimensions 1×5 μm for cone outer segments, 1×10 μm for rod outer segments and 1×1 μm for cone oil droplets) in the cell and recording the amount of light transmitted at each wavelength across the spectrum. A baseline scan was made subsequently in an identical fashion to the sample scan but from a tissue-free area of the preparation adjacent to the cell. The transmittance (ratio of sample to baseline signal) of the outer segment was calculated at each wavelength and converted to absorbance to give a pre-bleach spectrum. Each outer segment was then bleached with white light for 1–2 min and subsequent sample and baseline scans made to create a post-bleach spectrum (and thus confirm that any putative visual pigments were photolabile). Scans of oil droplets were converted from transmittance to absorptance prior to analysis and no bleaching was performed.

Spectra that satisfied established selection criteria (Levine and MacNichol, 1985; Hart et al., 1998) were retained for further analysis. Individual pre-bleach absorbance spectra were analyzed as described elsewhere (Hart, 2002), following the methods of MacNichol (MacNichol, 1986) and Govardovskii et al. (Govardovskii et al., 2000), to provide an estimate of the wavelength of maximum absorbance (λ_{max}) of each outer segment/visual pigment. The mean λ_{max} of a given visual pigment type was then calculated from these individual λ_{max} values. For display purposes, a mean pre-bleach absorbance spectrum was calculated by averaging acceptable individual (non-normalized) absorbance spectra and overlaid with a vitamin-A_1-based visual pigment template (Govardovskii et al., 2000) having the same λ_{max} value as this mean spectrum. Bleaching difference spectra were created by subtracting the post-bleach spectrum from the pre-bleach spectrum, and analyzed and averaged in the same way as the pre-bleach spectra. Microspectrophotometry of avian photoreceptors is challenging

because of the small size of the cone outer segments and their tendency to break off from the cell when the neural retina is separated from the retinal pigmented epithelium (RPE). Moreover, only one or two retinas were available from any of the six bowerbird species studied. Consequently, visual pigment absorbance spectra were not measured from the entire complement of cone types in all species, although oil droplet absorptance spectra were.

Oil droplet absorptance spectra were normalized to the maximum and long-wavelength offset absorptances obtained by fitting an 11-point unweighted ('boxcar') running average to the data (Hart et al., 1998; Hart, 2004). With the exception of the transparent T-type oil droplets found in one class of single cone, which had negligible absorptance across the spectrum, oil droplets were described by their cut-off wavelength (λ_{cut}), which is the wavelength of the intercept at the value of maximum measured absorptance by the line tangent to the oil droplet absorptance curve at half-maximum measured absorptance (Lipetz, 1984). For comparison with other studies, the wavelength corresponding to half-maximum measured absorptance (λ_{mid}) was also calculated (Lipetz, 1984).

Measuring the spectral transmittance of ocular media

The spectral transmittance of the intact anterior segment (cornea, aqueous humour and lens) of the right eye was measured using an Ocean Optics USB4000 spectrometer, a PX-2 pulsed xenon lamp (spectral output of 220–750 nm) and Spectrasuite data acquisition software (Ocean Optics, Dunedin, FL, USA). Measurements were made immediately after removal of the eye, before the cornea began to cloud. The anterior segment was dissected away and held in a fixed horizontal position. Light from the PX-2 lamp was delivered to the corneal surface along the optical axis using a quartz fibre optic light guide. Transmitted light was collected using another quartz fibre optic light guide and delivered to the spectrometer. Mean spectral transmittance was calculated from at least five spectral measurements collected from each eye.

Estimating relative cone proportions

Relative cone proportions were estimated by counting cone photoreceptor oil droplets in whole-mounted retinal tissue. Each right eye-cup was dissected into four quadrants (anterior–dorsal, anterior–ventral, posterior–dorsal and posterior–ventral) using the pecten as a marker for orientation (Hart, 2001a) and the tissue left in cold (4°C) PBS (340 mOsmol kg^{-1}) for 1 h to promote separation of the neural retina from the RPE. The tissue was then fixed for 2 min in 4% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer (pH 7.4) to reduce tearing during subsequent manipulation and prevent flattening when mounted. After washing in PBS, the retina was isolated from the sclera and mounted photoreceptor-side-up in PBS on a glass slide. Spacers made from strips of waxed paper tape were used to prevent the coverslip from squashing the retina and the preparation was sealed with nail varnish to prevent dehydration and movement of the specimen.

Retinal whole-mounts were viewed using bright field and UV-epifluorescence microscopy at a total magnification of ×1000 (Hart, 2001a). Each oil droplet was assumed to represent an individual cone cell and it was assumed that all cones possessed an oil droplet (with the exception of the accessory cones, which were not counted because we used counts of the oil droplets found in the principal member of the double cone to indicate double cone abundance). Microspectrophotometric measurements of these and other species (e.g. Bowmaker et al., 1997; Hart, 2002) have shown that each visual pigment/cone class is reliably associated with a given spectral type of oil droplet, as described above. P-, R- and Y-type oil droplets are readily distinguished from one another and from C- and T-type oil droplets using bright-field microscopy (see Fig. 1). Epifluorescent microscopy was used to discriminate between C- and T-type droplets, as C-type oil droplets fluoresce under UV illumination whereas T-type oil droplets do not. Oil droplets were counted from up to 10 fields of view (0.01 mm^2) selected haphazardly from within each of the four retinal quadrants. Counts were made from the middle of the quadrant, between the central retina and the periphery, where cone proportions approximate those averaged across the entire quadrant (Hart, 2001a), and converted to percentage abundance.

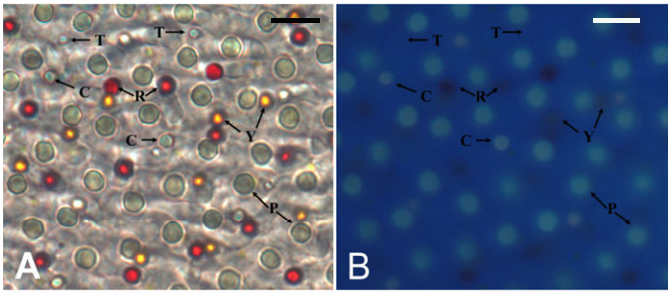


Fig. 1. Photomicrographs of satin bowerbird (*Ptilonorhynchus violaceus*) retinal tissue at ×1000 showing each class of coloured oil droplets. T, C, Y, R and P correspond to 'transparent' oil droplets in the inner segment of short-wavelength-sensitive 1 (SWS1) cones, 'colourless' droplets in short-wavelength-sensitive 2 (SWS2) cones, 'yellow' droplets in medium-wavelength-sensitive (MWS) cones, 'red' droplets in long-wavelength-sensitive (LWS) single cones, and 'pale' droplets in the primary member of double cones, respectively. Comparison between bright-field (A) and epifluorescent (B) microscopy aided discrimination between C- and T-type droplets; only C-type droplets are visible under both conditions. Scale bars, 10 μm.

RESULTS

Opsin sequence

Opsin cDNAs including all seven transmembrane domains (TMs) were sequenced for six species (see supplementary material Table S2 for sequence accession numbers). The lengths of the amino acid sequences translated from cDNA are 315 amino acids (aa) for SWS2, 285 aa for Rh2, 334 aa for LWS and 324 aa for Rh1. The gDNA sequence for LWS and Rh2 opsin genes was obtained from 12 species and contains most of the exonic sequence, including all of TM 1, 2 and 4–7, partial TM 3, and all key amino acid sites listed above. A relatively limited gDNA sequence was obtained for SWS2, despite efforts to optimize primer design and PCR conditions; however, successfully amplified portions include TM 5–7 and spectrally significant sites 261, 269 and 292. The lengths of amino acid sequence translated from gDNA are 87 aa for SWS2, 273 aa for Rh2 and 276 aa for LWS, except for Vogelkop and spotted bowerbird LWS opsins, which were 270 and 222 aa long, respectively.

Comparison of translated opsin amino acid sequences revealed little variation between bowerbirds (Tables 2, 3). Most of the variation involves functionally conserved substitutions at sites located outside the chromophore binding pocket (Table 3). The two most basal species, green catbird and toothbilled bowerbird, differed most from consensus opsin sequences. The only variation at a key opsin tuning site was an interspecific valine-52-leucine (V52L) SWS2 opsin polymorphism. Four of the six species for which cDNA was sequenced have V52, and green catbird and toothbilled bowerbird have L52. Absorptance measurements could not be

Table 2. Comparison of visual pigment opsin amino acid residues at known spectral tuning sites and visual pigment spectral absorbance characteristics measured using microspectrophotometry

	Visual pigment and corresponding opsin									
Species	SWS1 λ_{max}	Violet	SWS2 λ_{max}	Blue	Rh2 λ_{max}	Green	LWS λ_{max}	Red	Rh1 λ_{max}	Rod
Ailuroedus crassirostris	406	.		52L	507	.	562	.	501	.
Scenopooetes dentirostris		.		52L		.	563	.	506	.
Sericulus chrysocephalus	408	.		.		.	563	.	503	.
Ptilonorhynchus violaceus	410	.	454	.	511	.	562	.	503	.
Chlamydera nuchalis	404	.		.	503	.	564	.	503	.
Chlamydera maculata		.		.		.		.	505	.
Chlamydera cerviniventris		.		*261F, 269S, 292S		.		.		
Chlamydera gutatta		.		.		.		.		
Amblyornis macgregoraie		.		.		.		.		
Amblyornis subalaris		.		.		.		.		
Amblyornis papuensis		.		.		.		.		
Amblyornis inornatus		.		.		.		.		
Prionodura newtoniana		.		.		.		.		

Species were compared to consensus amino acid residues at tuning sites. A dot indicates total agreement with consensus and a blank space indicates no data for those sites. Sequence data were available for all violet (SWS1), green (Rh2) and red (LWS) opsin tuning sites for all 13 species. All blue (SWS2) and rod opsin tuning sites were sequenced for the six species for which retinal tissues were available but only three SWS2 tuning sites (*) were available for the remaining seven species. The only amino acid polymorphism detected for any opsin is at SWS2 site 52. Data for the SWS1 opsin come from Zwiers (Zwiers, 2009) (P. Zwiers, personal communication). λ_{max}, mean wavelength (nm) of maximum absorbance of the visual pigment. Amino acid numbering follows that of bovine rod opsin.

Consensus residues are as follows: violet – 46V, 86C, 90S, 93T, 116A; blue – 46L, 49A, 52V, 91S, 93T, 94A, 116A, 122M, 164G, 207L, 261F, 269S, 292S; green – 122Q, 222S, 295S; red – 164S, 181H, 261Y, 269T, 292A; and rod – 122E, 222C, 295A.

obtained for the SWS2 cone outer segment from either toothbilled bowerbird or green catbird, and thus we were not able to assess whether L52 changes spectral sensitivity compared with a pigment with V52. However, the lack of a change in amino acid polarity corresponding with a V52L substitution (both residues are non-polar) suggests it is probably a functionally conservative polymorphism. Additionally, comparison of opsin sequences between bowerbirds and other bird species, including canary, *Serinus canaria* (Das et al., 1999), zebra finch, *Taeniopygia guttata* (Yokoyama et al., 2000), chicken, *Gallus gallus* (Okano et al., 1992), and pigeon, *Columbia livia* (Kawamura et al., 1999), revealed no other previously unreported residues at known or suspected tuning sites.

Microspectrophotometry of visual pigments and oil droplets

The available data suggest that the spectral characteristics of the visual pigments and oil droplets are very similar across all six bowerbird species tested (Tables 4, 5). They possess a single class of rod photoreceptor that has an MWS visual pigment with a mean λ_{max} between 501 and 506 nm; four subtypes of single cone photoreceptor that are maximally sensitive to either violet- (VS), short- (SWS), medium- (MWS) or long-wavelengths (LWS); and also LWS double cones, both the principal and accessory members of which contain the same LWS visual pigment as the LWS single cones (Figs 2, 3 show absorbance and difference spectra from visual pigments in satin bowerbird, which are very similar to visual pigment spectra from other bowerbird species). Mean λ_{max} values for the VS, SWS, MWS and LWS visual pigments across the six species of bowerbird studied ranged from 404–410 nm, 454 nm, 503–511 nm to 558–568 nm, respectively.

VS single cones contained a transparent T-type oil droplet that had negligible absorptance (mean <0.04) across the spectrum. Each of the other three single cone types and the principal member of the double cone contained a pigmented/coloured oil droplet with type-specific spectral absorptance characteristics (Figs 4, 5, Table 5).

Table 3. Variation in SWS2, RH2, LWS and RH1 opsin sequences

Opsin	SWS2								RH2		LWS				RH1
Amino acid position	38	52	54	59	112	121	331	332	108	165	11	99	213	220	217
Consensus amino acid	R	V	V	V	V	G	E	D	I	I	V	I	F	I	L
Ailuroedus crassirostris	T	L	I	I	I	T	D	E	V	V	I	V	.	.	S
Scenopooetes dentirostris	T	L	I	.	I	.	D	.	V	.	.	V	.	V	S
Sericulus chrysocephalus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Ptilonorhynchus violaceus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chlamydera nuchalis	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Chlamydera maculata	.	.	.	.	.	.	.	.	.	.	.	.	L	.	.
Chlamydera cerviniventris									.	.		.	.	.	
Chlamydera gutatta									.	.		.	.	.	
Amblyornis macgregoraie									V	.		V	.	.	
Amblyornis subalaris									V	.		V	.	.	
Amblyornis papuensis									V	.		V	.	.	
Amblyornis inornatus									V	.		V	.	.	
Prionodura newtoniana									V	.		V	.	.	

These sites have not been associated with spectral tuning. Dots indicate agreement with consensus sequence. Absence of dot or residue indicates that there are no data for that particular site. Amino acid numbering follows that of bovine rod opsin.

Table 4. Characteristics of visual pigments measured using microspectrophotometry

	Single cones				Double cones		
	VS	SWS	MWS	LWS	Principal	Accessory	Rods
Satin – *Ptilonorhynchus violaceus*							
Mean λ_{max} of pre-bleach spectra (nm)	410.2±2.8	454.2±1.4	511.2±0.4	561.7±6.2	562.1±3.4	562.7±2.5	503.1±1.2
λ_{max} of mean pre-bleach spectrum (nm)	410.3	456.0	513.8	560.2	561.9	561.9	503.0
Mean λ_{max} of difference spectra (nm)	410.4±9.9	450.3±4.7	509.1±18.0	562.8±6.6	563.7±4.2	562.8±2.2	504.7±0.5
λ_{max} of mean difference spectrum (nm)	408.5	452.3	514.4	560.7	563.1	562.1	504.7
N	3	4	2	3	4	5	11
Great – *Chlamydera nuchalis*							
Mean λ_{max} of pre-bleach spectra (nm)	–	–	502.6±8.7	–	563.6±6.1	567.2±0.1	502.8±1.3
λ_{max} of mean pre-bleach spectrum (nm)	404.4	–	502.7	564.7	562.0	566.8	502.6
Mean λ_{max} of difference spectra (nm)	–	–	506.0±9.8	–	562.1±3.8	574.5±3.1	505.7±1.8
λ_{max} of mean difference spectrum (nm)	409.5	–	508.4	565.8	562.6	572.6	505.7
N	1	–	2	1	10	2	4
Catbird – *Ailuroedus crassirostris*							
Mean λ_{max} of pre-bleach spectra (nm)	–	–	506.6±3.3	–	561.6±3.9	–	501.4±1.8
λ_{max} of mean pre-bleach spectrum (nm)	405.9	–	506.0	568.2	562.0	565.9	501.0
Mean λ_{max} of difference spectra (nm)	–	–	506.7±4.9	–	559.8±11.1	–	504.6±1.6
λ_{max} of mean difference spectrum (nm)	392.5	–	506.8	570.4	560.8	570.1	505.0
N	1	–	2	1	3	1	3
Regent – *Sericulus chrysocephalus*							
Mean λ_{max} of pre-bleach spectra (nm)	–	–	–	–	562.8±3.2	563.9±4.1	503.5±0.8
λ_{max} of mean pre-bleach spectrum (nm)	408.2	–	–	–	563.1	564.1	503.3
Mean λ_{max} of difference spectra (nm)	–	–	–	–	564.6±4.3	561.8±7.5	506.0±1.6
λ_{max} of mean difference spectrum (nm)	404.9	–	–	–	563.4	562	505.3
N	1	–	–	–	11	4	9
Toothbilled – *Scenopoetes dentirostris*							
Mean λ_{max} of pre-bleach spectra (nm)	–	–	–	–	558.4±0.8	–	505.9±0.2
λ_{max} of mean pre-bleach spectrum (nm)	–	–	–	562.9	557.9	562.8	506.3
Mean λ_{max} of difference spectra (nm)	–	–	–	–	557.8±1.6	–	506.3±2.3
λ_{max} of mean difference spectrum (nm)	–	–	–	565.3	557.6	561.9	507.2
N	–	–	–	1	3	1	3
Spotted – *Chlamydera maculata*							
Mean λ_{max} of pre-bleach spectra (nm)	–	–	–	–	–	–	504.5±1.7
λ_{max} of mean pre-bleach spectrum (nm)	–	–	–	–	–	–	504.2
Mean λ_{max} of difference spectra (nm)	–	–	–	–	–	–	505.2±1.9
λ_{max} of mean difference spectrum (nm)	–	–	–	–	–	–	504.7
N	–	–	–	–	–	–	2

Values are means ± 1 s.d.

Almost invariably, the coloured oil droplets in cones located in the ventral retina had λ_{cut} values at longer wavelengths than the same oil droplet type in cones located in the dorsal retina. Across all six species, mean λ_{cut} values for the C-, Y-, R- and P-type droplets in the dorsal retina were 418–428, 508–515, 558–571 and 412–425 nm, respectively, whereas in the ventral retina they were 424–441, 516–523, 566–573 and 495–503 nm, respectively. The accessory member of the double cone pair contained an oil droplet only in cells located in the ventral retina. In the catbird and spotted bowerbird, the mean peak absorptance of the A-type oil droplets was very low (0.05–0.08), but in the other four species the mean peak absorptance was considerably greater (0.13–0.38) and λ_{cut} values ranged from 456 to 492 nm. Instead of an oil droplet, the ellipsoid region of the inner segment of accessory cones located in the dorsal retina contained very low levels of pigment that had spectral absorptance characteristics similar to those of the A-type oil droplets in the ventral retina.

Ocular media

The spectral transmittances (250–800 nm) of the ocular media of five species of bowerbird were measured in air using a spectrometer. Multiple spectra from individual eyes and birds were normalized, averaged together and the resultant mean spectrum for each species was interpolated to 1 nm intervals, smoothed with an 11-point unweighted running average and normalized again. In every case, the spectrum displayed a broad plateau of high transmittance from 800 nm down to approximately 400 nm (Fig. 6). Below 400 nm, the transmittance dropped rapidly and wavelengths below approximately 300 nm were not transmitted. For comparison with other studies, the wavelengths of 0.5 normalized transmittance ($\lambda T_{0.5}$) of the ocular media were calculated for the catbird, satin, regent, great and spotted bowerbirds, and were 340, 343, 349, 349 and 352 nm, respectively.

Cone proportions

Based on subjective assessment (the small sample size precluded statistical analysis), cone proportions and their distribution across the retina appear relatively similar across species; however, more data are required to test this properly (Fig. 7). The relative percentage abundance of the different oil droplet (cone) types for the whole retina across five species was: T-type (VS), 5.6–8.2%; C-type (SWS), 9.6-15.6%; Y-type (MWS), 17.6–21.2%; R-type (LWS), 17–22%; and P-type (double cones), 38.9–46.6%. The spotted bowerbird was excluded from this comparison because the RPE did not separate from much of the neural retina and cone counts could not be made for all retinal quadrants (see supplementary material Table S3 for cone count data by quadrant for six species).

Table 5. Characteristics of coloured oil droplets measured *via* microspectrophotometry

	Single cones							Double cones			
		C-type		Y-type		R-type		P-type		A-type	
	T-type	D	V	D	V	D	V	D	V	D	V
Satin – *Ptilonorhynchus violaceus*											
Mean λ_{cut} of absorptance spectra (nm)	–	423.3±1.6	437.2±6.4	514.4±3.9	521.3±2.1	567.6±2.8	571.7±1.2	416.2±4.2	494.9±4.7	–	–
λ_{cut} of mean absorptance spectrum (nm)	–	423.2	442.5	514.6	521.4	567.3	570.5	415.3	495.6	–	485.7
Mean λ_{mid} of absorptance spectra (nm)	–	435.3±1.3	460.0±7.1	533.6±4.1	540.6±2.1	590.6±2.6	595.8±1.6	439.2±3.8	509.4±4.0	–	–
λ_{mid} of mean absorptance spectrum (nm)	–	435.2	460.0	534.1	540.7	590.7	594.6	439.3	510.5	–	501.6
Mean maximum transverse absorptance	0.03±0.01	0.35±0.08	0.16±0.06	0.56±0.08	0.52±0.06	0.72±0.06	0.54±0.12	0.49±0.09	0.43±0.13	0.06±0.08	0.38
Mean diameter (μm)	2.1±0.2	2.2±0.3	2.0±0.2	2.6±0.2	2.4±0.3	3.0±0.1	3.0±0.0	3.6±0.3	2.7±0.3	i/s	1.25
N	13	13	10	15	9	16	11	20	14	4	1
Great – *Chlamydera nuchalis*											
Mean λ_{cut} of absorptance spectra (nm)	–	421.0±3.9	425.3±4.3	515.0±3.7	519.4±2.7	567.7±2.9	569.9±1.7	420.1±5.1	502.5±6.9	–	489.1±2.1
λ_{cut} of mean absorptance spectrum (nm)	–	422.2	425.7	513.4	516.4	567.8	569.8	418.7	503.3	–	490.1
Mean λ_{mid} of absorptance spectra (nm)	–	440.4±0.3	447.4±2.3	530.6±5.0	537.2±2.8	589.7±3.4	592.4±2.1	445.9±4.9	516.6±6.2	–	501.8±2.6
λ_{mid} of mean absorptance spectrum (nm)	–	440.4	447.6	530.5	535.4	589.9	592.4	445.8	517.9	–	502.3
Mean maximum transverse absorptance	0.03±0.01	0.33±0.02	0.41±0.08	0.80±0.06	0.69±0.15	0.84±0.03	0.80±0.05	0.51±0.18	0.74±0.08	0.02	0.33±0.10
Mean diameter (μm)	2.2±0.2	2.8±0.3	2.9±0.2	3.8±0.4	3.4±0.3	4.2±0.2	3.6±0.2	3.7±0.4	3.6±0.3	i/s	1.9±0.2
N	8	4	9	10	9	10	9	14	17	1	10
Green catbird – *Ailuroedus crassirostris*											
Mean λ_{cut} of absorptance spectra (nm)	-	420.7±2.5	440.7±4.3	508.3±1.6	516.1±2.7	558.4±3.9	566.0±1.7	411.5±4.3	500.3±2.0	–	–
λ_{cut} of mean absorptance spectrum (nm)	–	421.6	443.7	508.2	516.2	558.3	565.6	411.2	500.7	–	–
Mean λ_{mid} of absorptance spectra (nm)	–	438.4±2.8	457.6±3.0	525.7±1.5	536.6±2.4	579.7±4.3	588.1±2.3	441.0±3.0	513.8±1.7	–	–
λ_{mid} of mean absorptance spectrum (nm)	–	437.9	457.8	525.4	536.5	579.9	587.7	440.1	514.0	–	–
Mean maximum transverse absorptance	0.04±0.03	0.60±0.12	0.22±0.04	0.73±0.09	0.48±0.10	0.81±0.05	0.63±0.11	0.39±0.16	0.56±0.03	0.02	0.05±0.02
Mean diameter (μm)	2.2±0.3	2.8±0.36	2.2±0.3	2.9±0.2	3.0±0.3	3.4±0.4	3.5±0.0	4.2±0.3	3.4±0.2	i/s	1.9±0.2
N	8	9	9	12	10	11	10	13	10	1	6
Regent – *Sericulus chrysocephalus*											
Mean λ_{cut} of absorptance spectra (nm)	–	417.7±3.2	436.6±2.9	510.7±3.2	518.9±1.7	566.7±1.4	573.4±1.0	418.5±6.5	500.5±3.6	–	491.6±0.8
λ_{cut} of mean absorptance spectrum (nm)	–	417.7	438.7	511.3	519.5	566.3	573.1	419.9	500.2	–	491.8
Mean λ_{mid} of absorptance spectra (nm)	–	431.0±2.1	454.6±4.0	527.7±4.9	540.6±1.5	589.3±1.4	597.5±1.1	445.3±4.2	517.5±4.5	–	505.4±0.4
λ_{mid} of mean absorptance spectrum (nm)	–	430.8	455.8	528.1	540.5	589.1	597.4	445.8	517.4	–	504.2
Mean maximum transverse absorptance	0.04±0.03	0.33±0.13	0.10±0.02	0.62±0.10	0.36±0.07	0.80±0.07	0.45±0.04	0.60±0.15	0.34±0.07	0.08±0.06	0.13±0.12
Mean diameter (μm)	2.0±0.4	2.0±0.1	1.7±0.3	2.6±0.3	2.6±0.3	3.4±0.2	2.8±0.3	3.9±0.3	3.0±0.1	i/s	1.1±0.1
N	8	10	6	11	10	10	10	21	11	3	4
Toothbilled – *Scenopoetes dentirostris*											
Mean λ_{cut} of absorptance spectra (nm)	–	423.7±2.3	423.8±7.1	513.9±2.9	522.9±2.2	566.7±1.3	569.8±1.4	417.8±1.7	501.1±2.1	–	–
λ_{cut} of mean absorptance spectrum (nm)	–	425.3	424.2	511.7	523.2	566.2	569.7	417.8	501.1	–	455.7
Mean λ_{mid} of absorptance spectra (nm)	–	437.8±1.2	451.3±4.3	532.2±4.3	542.0±2.8	589.0±1.3	592.5±1.7	435.1±3.0	514.7±2.1	–	–
λ_{mid} of mean absorptance spectrum (nm)	–	437.5	449.8	531.2	542.2	588.6	592.4	434.7	514.8	–	494.4
Mean maximum transverse absorptance	0.04±0.03	0.33±0.12	0.49±0.13	0.60±0.15	0.75±0.07	0.73±0.11	0.81±0.05	0.58±0.12	0.77±0.06	0.03	0.15
Mean diameter (μm)	2.0±0.0	2.3±0.3	2.5±0.4	2.9±0.2	3.1±0.3	3.6±0.2	3.5±0.2	3.9±0.2	3.7±0.3	i/s	1.8
N	6	10	11	10	10	10	10	13	10	1	1
Spotted – *Chlamydera maculata*											
Mean λ_{cut} of absorptance spectra (nm)	–	427.7±3.8	436.3±4.8	514.7±2.8	519.9±1.9	570.9±1.5	570.8±1.7	425.1±4.2	496.6±0.1	–	–
λ_{cut} of mean absorptance spectrum (nm)	–	428.0	438.8	513.9	520.1	570.9	570.5	425.5	496.5	–	–
Mean λ_{mid} of absorptance spectra (nm)	–	448.7±2.1	455.6±3.1	532.5±3.7	540.2±1.9	594.9±1.8	594.8±1.3	448.2±2.4	510.9±2.1	–	–
λ_{mid} of mean absorptance spectrum (nm)	–	448.5	456.4	532.4	540.2	594.8	594.7	448.3	511.1	–	–
Mean maximum transverse absorptance	0.03±0.01	0.41±0.06	0.25±0.08	0.71±0.07	0.55±0.06	0.80±0.04	0.64±0.04	0.46±0.13	0.48±0.07	–	0.08±0.01
Mean diameter (μm)	2.2±0.2	2.6±0.1	2.5±0.1	3.3±0.4	3.0±0.2	4.0±0.1	3.4±0.3	3.8±0.4	3.4±0.2	–	1.5±0
N	4	9	10	10	10	10	10	10	10	–	–

Values are means ± 1 s.d.
D, dorsal; i/s, diffuse pigment in inner segment measured, no oil droplet; V, ventral.

DISCUSSION

The aims of this study were to characterize bowerbird visual systems and compare sensitivity between species to determine whether there might be tuning differences that are related to interspecific variation in display colour. We detected intraretinal variations in oil droplet spectral transmittance and relative cone abundance. We also found that bowerbird ocular media transmit significantly more UV wavelengths than those of most other species with a VS-type SWS1 visual pigment. However, overall these data reveal a generally low level of interspecific variation in visual sensitivity, which suggests that there is no relationship between spectral tuning and species divergence in display colour.

Visual pigments

We show that visual pigments are spectrally similar among bowerbirds based on comparison of spectral absorptance measurements made using microspectrophotometry and opsin sequence data. The λ_{max} values for VS, Rh1, LWS and Rh2 pigments were the same or very close (within experimental error) among the species we examined. SWS2 λ_{max} was obtained only for the satin bowerbird, however, the absence of spectrally important opsin

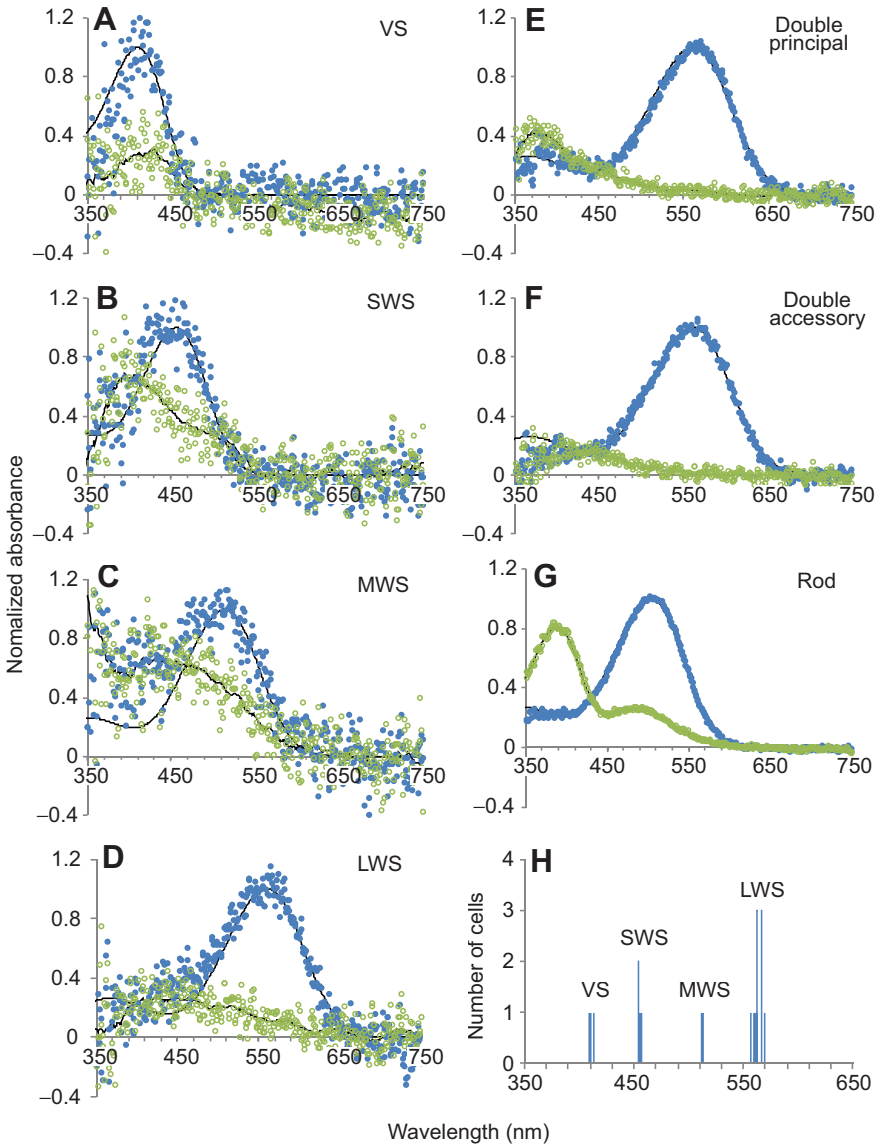


Fig. 2. (A–G) Normalized mean pre- (filled circles) and post-bleach (empty circles) spectra of visual pigments measured using microspectrophotometry from satin bowerbird (*P. violaceus*) photoreceptor outer segments. Pre-bleach spectra are overlayed with best-fit rhodopsin templates. Post-bleach spectra are fitted with variable-point running average. (H) Histogram shows the spectral distribution of the wavelength of maximum absorbance (λ_{max}) for individual photoreceptor cell outer segments that were used to generate the mean spectra. The data for LWS visual pigment distribution includes measurements from LWS single cones and both the principal and accessory members of double cones. SWS, short-wavelength-sensitive; VS, violet sensitive.

sequence variation between the satin bowerbird and the five other species for which all SWS2 opsin tuning sites were sequenced indicates that all six species probably share the same λ_{max}. Likewise, λ_{max} values obtained for the other visual pigments from a subset of species are generalizable to all species having the same amino acid residues at key opsin sites. These include the opsins we sequenced in this study and the previously examined SWS1 opsin (VS-type) that has the same critical amino acid residues (C86, S90 and T93) across 15 bowerbird species (Zwiers, 2009). Furthermore, the λ_{max} values for all five pigments are consistent with the predicted sensitivity based on opsin sequence [for a review of opsin tuning sites and spectral significance of various amino acids, see Yokoyama (Yokayama, 2008)]. The only variation at a key opsin site that we uncovered is an interspecific SWS2 opsin L52A substitution.

The presence of L52 in the SWS2 opsin has not been reported in any other species. Other substitutions at SWS2 site 52 that involve a change in amino acid polarity have been demonstrated to shift sensitivity by as much as 12 nm (Yokoyama and Tada, 2003), but given that alanine and leucine are both non-polar residues it is unlikely that the A52L polymorphism in bowerbirds is spectrally significant. Furthermore, neither the green catbird nor the toothbilled bowerbird possesses obvious colour characteristics that suggest any possible variation in SWS2 sensitivity related to signal design. For example, neither species has a prominent signal element in the SWS2 spectral range, such as peak spectral reflectance of plumage or decoration: toothbilled males have drab olive/brown plumage and use whitish green leaves for decorations, and green catbirds have predominantly green plumage and they do not collect decorations. Also, there is no evidence that colour displays play a particularly important role in green catbird courtship [for description of courtship, see Frith et al. (Frith et al., 2004)]. More significantly, there is no interspecific variation in visual pigment sensitivity among the most colourful bowerbird species we studied in which the importance of display colour has been demonstrated.

Coloured oil droplets

All classes of coloured oil droplets had longer wavelength λ_{cut} values in the ventral retina compared with the dorsal retina in most species. The difference was most pronounced in C-type droplets of the SWS cones and especially in P-type droplets of the principal member of double cones. This pattern of intraretinal variation has also been observed in other species, which suggests that it may be common

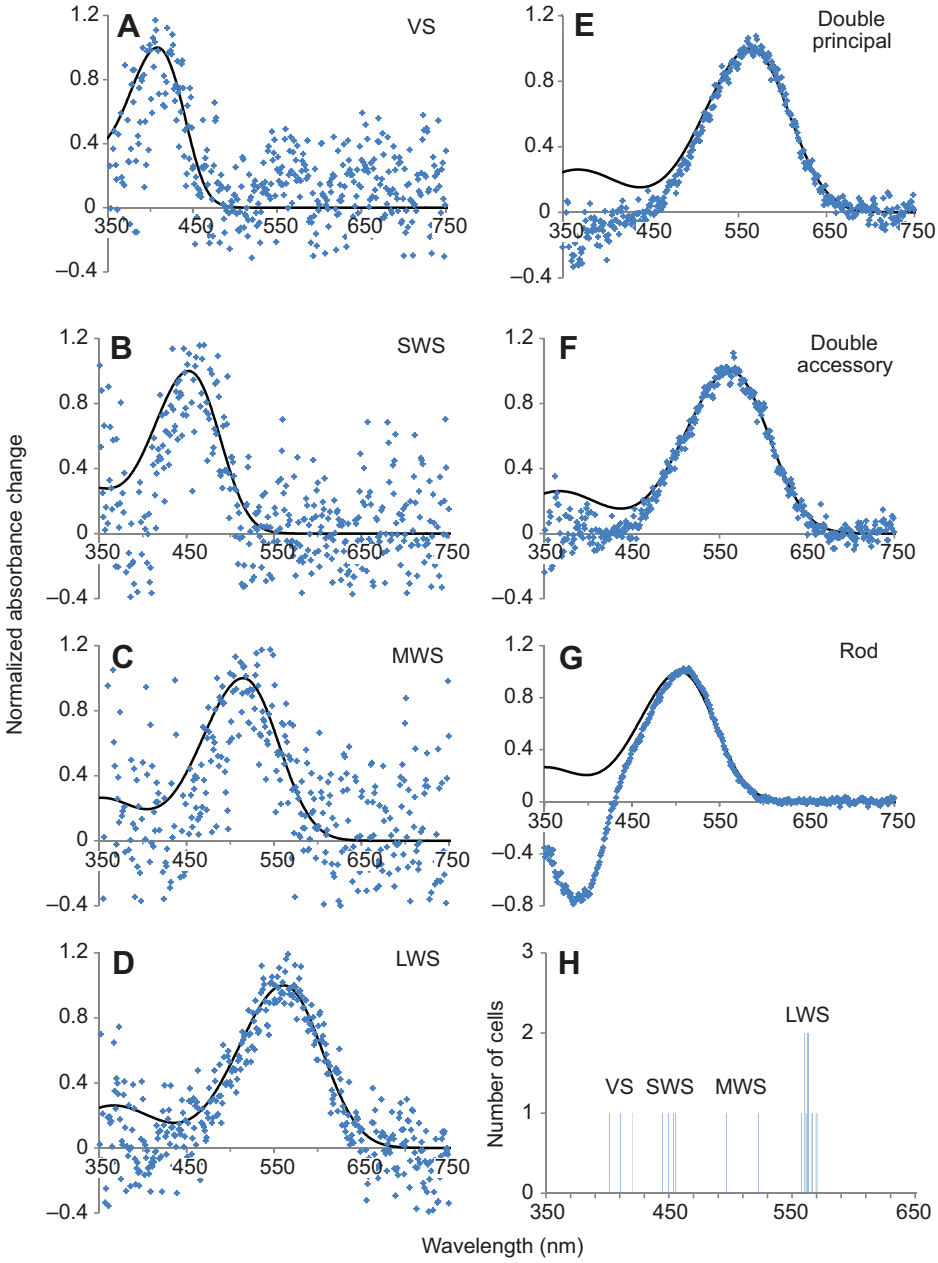


Fig. 3. (A–G) Normalized mean bleaching difference spectra (symbols) and best-fitted rhodopsin visual pigment templates (lines) for visual pigments in the satin bowerbird (*P. violaceus*). The difference spectra represent the change in absorbance of the photoreceptor outer segment following bleaching with white light. (H) Histogram shows the spectral distribution of λ_{max} for each visual pigment difference spectra that were used to generate the mean spectra.

across many bird taxa (e.g. Hart, 2004; Hart et al., 2006). Experimental evidence suggests that the more dense pigmentation of oil droplets in cones of the ventral retina may function to buffer the greater intensity of downwelling light that they receive compared with cones in the dorsal retina (Hart et al., 2006). Because of this intraretinal variation in coloured oil droplet spectral absorptance, the overall spectral sensitivity of the dorsal and ventral regions will differ. In particular, SWS, MWS and LWS cones in the ventral retina may have substantially greater photon catch than in the dorsal retina, providing that outer segment lengths and visual pigment density are identical (Fig. 8).

In contrast to the typical pattern of intraretinal variation, however, the λ_{cut} values of C-type droplets did not differ between the ventral and dorsal SWS2 cones in great or toothbilled bowerbirds. Rather, the C-type droplet λ_{cut} values in these two species were similar to values in the dorsal SWS2 cones of all species (i.e. approximately 12 nm shorter wavelength than in ventral cones). Consequently, ventral SWS2 cones may have substantially greater photon catch in great and toothbilled bowerbirds compared with the other four species that were examined (providing that outer segment lengths and visual pigment density are identical). This may provide an important benefit to toothbilled bowerbirds because they occupy dimly lit closed-canopy rain-forest habitats that are typically characterized by relatively low-intensity short-wavelength light (Endler, 1993). However, great bowerbirds occupy open and sunny habitat where there is no obvious need for increased SWS2 cone sensitivity and thus, no common explanation for the presence of this trait in both species is readily apparent.

Ocular media

Ocular transmission of UV determines the short wavelength limit of visual ability, and an increase in UV transmission was clearly important in the evolution of the derived avian UVS visual system from the ancestral VS visual system. However, it remains unclear whether the SWS1 pigment shifted from VS to UVS before or following reduction of UV blocking ocular pigments. Of the bird

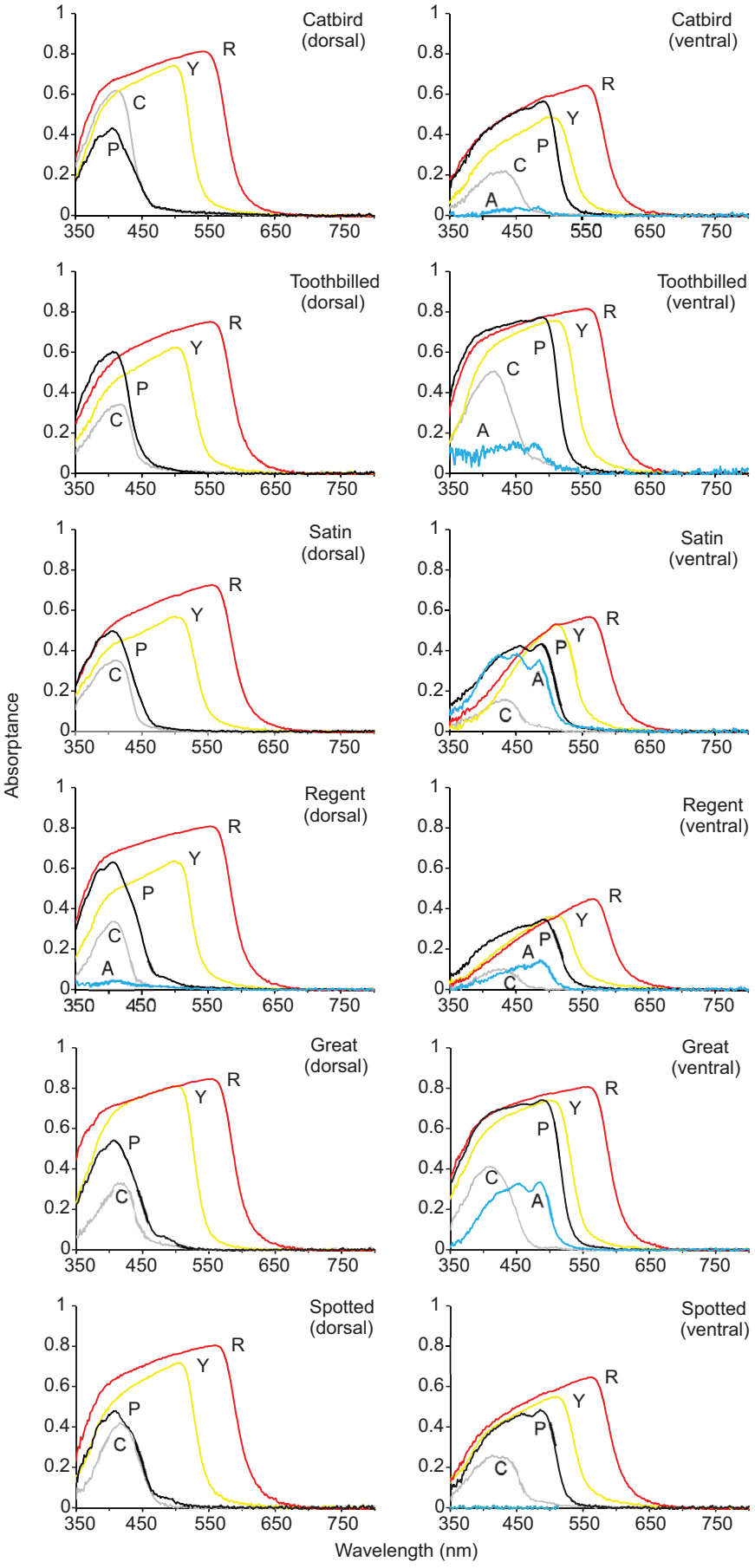


Fig. 4. Mean absorptance spectra of cone photoreceptor oil droplets for dorsal and ventral retina for six species. C, Y, R, P and A correspond to 'transparent' oil droplets in the inner segment of SWS1 cones, 'colourless' droplets in SWS2 cones, 'yellow' droplets in MWS cones, 'red' droplets in LWS single cones, 'pale' droplets in the primary member of double cones, and A droplets in the accessory member of the double cone, respectively.

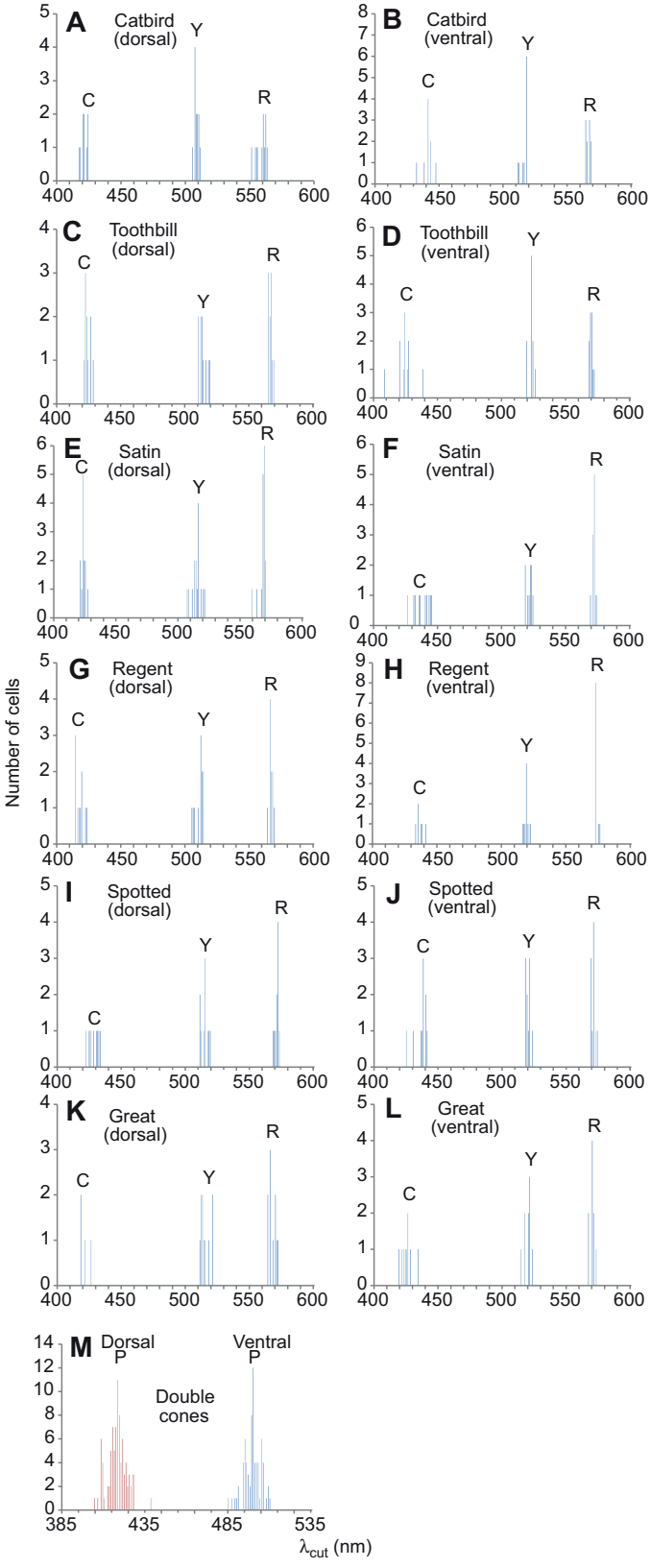


Fig. 5. (A–L) Histograms show the spectral distribution of the cut-off wavelength (λ_{cut}) for oil droplets located in single cones that were used to generate mean spectra for the dorsal and ventral retina for six species. (M) Histogram showing the spectral distribution of λ_{cut} for P-type oil droplets of the principal member of double cones for dorsal and ventral retina.

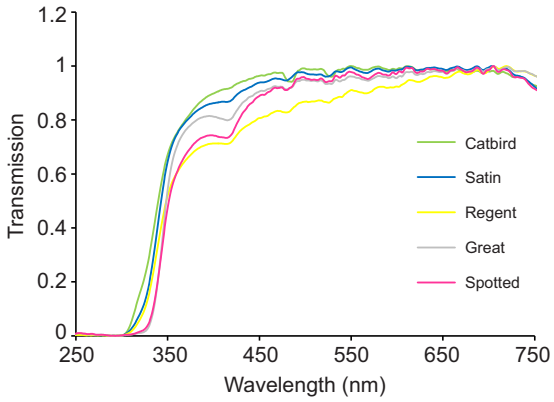
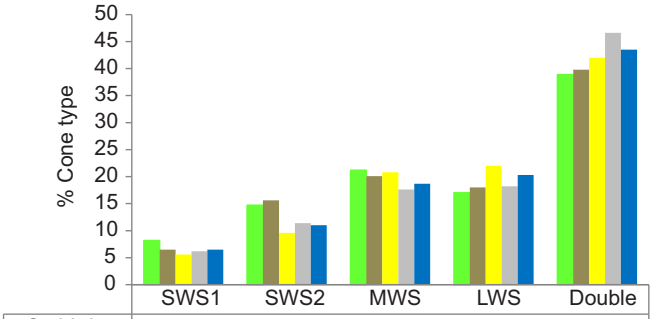


Fig. 6. Ocular media transmittance spectra for five species. The wavelengths of 0.5 normalized transmittance ($\lambda T_{0.5}$) of the ocular media of the green catbird (*Ailuroedus crassirostris*), and satin (*Ptilonorhynchs violaceus*), regent (*Sericulus chrysocephalus*), great (*Chlamydera nuchalis*) and spotted bowerbird (*Chlamydera maculata*) were 340, 343, 349, 349 and 352 nm, respectively.

species for which ocular transmission data are available (see Hastad et al., 2009), all species with UVS pigments have $\lambda T_{0.5} \leq 343$ nm whereas most species with VS pigments have $\lambda T_{0.5} \geq 359$ nm. Hastad et al. (Hastad et al., 2009) calculated that a shift from $\lambda T_{0.5}$ 365 nm to $\lambda T_{0.5}$ 338 nm increases SWS1 photon catch by approximately 40% with a VS pigment and 59% with a UVS pigment. In contrast, a shift from VS to UVS pigment in the presence of $\lambda T_{0.5}$ 365 nm has very little effect on SWS1 photon catch. Based on these calculations, they hypothesized that a loss of UV-filtering ocular pigment likely preceded and predisposed the evolution of the UVS pigment. Intriguingly, considering that all passerine lineages younger than bowerbirds have UVS-type visual systems, the unusually low $\lambda T_{0.5}$ values of bowerbirds (between 340 and 352 nm) suggest the possibility that they represent the proposed transitional link from a VS to a UVS visual system.

Regardless of the evolutionary trajectory of SWS1 sensitivity in bowerbirds, their relatively UV transparent ocular media should enable communication over a wider spectrum than many other species with the VS-type pigment. Moreover, some of the most prominent sexual displays of multiple bowerbird species have strong reflectance at relatively short wavelengths of UV that could potentially function as a signal element. For example, the spectacular plumage crests of great, spotted and western bowerbirds have high UV reflectance below 363 nm (Zwiers, 2009), and courting males usually display their crest under UV-rich light conditions. We calculated that great bowerbird VS photon catch of crest reflectance under typical light conditions during courtship is 13% higher than it would be if the great bowerbird instead possessed ocular media characteristic of many other birds with a similar VS visual pigment (Fig. 9). This indicates that a previous spectral analysis of bowerbird colour display evolution (Endler et al., 2005) underestimated their UV sensitivity and potential signaling ability because their visual performance was simulated using optical parameters of a typical VS-type system (i.e. ~$\lambda T_{0.5}$ 362 nm). Furthermore, the greater UV sensitivity that we detected would likely enhance perceived colour contrast of the crest, as males typically display their crest during courtship against a low UV-reflecting background [for characterization of spectral conditions see Endler et al. (Endler et al., 2005)], thereby making it more conspicuous and potentially more attractive. However, behavioral studies are necessary to determine whether UV reflectance of plumage crest is an important signal



	SWS1	SWS2	MWS	LWS	Double
Catbird	8.2	14.7	21.2	17	38.9
Toothbilled	6.5	15.6	20.1	18	39.8
Regent	5.6	9.6	20.8	22	42
Great	6.2	11.4	17.6	18.2	46.6
Satin	6.5	11	18.7	20.3	43.5

Fig. 7. Relative proportion of oil droplet types across the whole eye of green catbird (*Ailuroedus crassirostris*; green), and toothbilled (*Scenopooetes dentirostris*; brown), regent (*Sericulus chrysocephalus*; yellow), great (*Chlamydera nuchalis*; grey) and satin bowerbird (*Ptilonorhynchs violaceus*; blue). Oil droplets were used as a proxy to estimate relative cone proportions. Spotted bowerbird (*Chlamydera maculata*) is not included because counts were available for only a single retinal quadrant (see supplementary material Table S3 for percent data by quadrant for all six species).

element and the potential significance of the increased VS photon catch to signal perception and mate choice.

There is controversy over the hypothesis that UV signal elements are favored by selection because they provide a 'safe' channel for sexual signaling that some predators cannot easily detect (e.g. Bennett and Cuthill, 1994; Kevan et al., 2001; Hausmann et al., 2003; Hastad et al., 2005; Stevens and Cuthill, 2007). Most recently, Odeen et al. (Odeen et al., 2011) provide evidence for possible co-

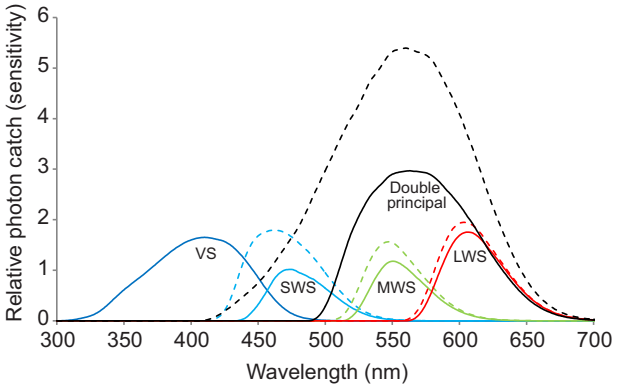


Fig. 8. Calculated relative photon catch for dorsal (dashed lines) and ventral (solid lines) cone photoreceptors in the satin bowerbird (*P. violaceus*). For each cone class, cones in the dorsal retina have greater photon catch and lower λ_{max} than cones in the ventral retina, except for the SWS1 cone, which has a transparent droplet that transmits nearly all light. Visual pigment spectral absorptance was modelled using a mathematical template based on the appropriate λ_{max} (Govardovskii et al., 2000). Outer segment length was assumed to be 16 μm (Morris and Shorey, 1967) with end-on specific absorbance of 0.015 μm^{-1} (Bowmaker, 1977). The spectral absorptance of visual pigments for dorsal and ventral retina were multiplied by the spectral transmittance of the ocular media and coloured oil droplets, and the cross-sectional area of the oil droplets (see Table 5). Coloured oil droplets were considered to act as long-pass cut-off filters, whereby they are assumed to block all wavelengths below λ_{cut} (Hart and Vorobyev, 2005).

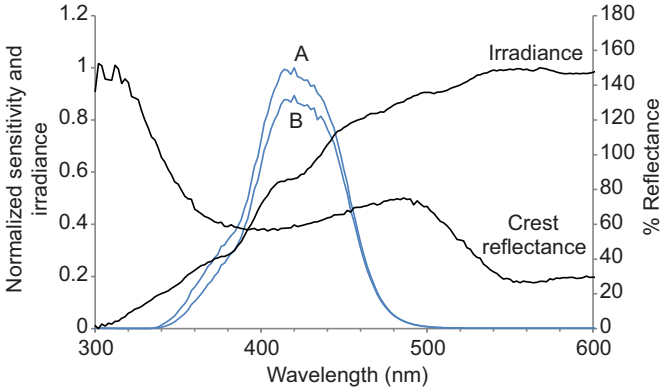


Fig. 9. Calculated relative photon catch of the great bowerbird (*C. nuchalis*) SWS1 single cone photoreceptor combined with ocular transmission of (A) great bowerbird ($\lambda T_{0.5}$ 348 nm) and (B) a typical VS-type bird ($\lambda T_{0.5}$ 363 nm), with respect to plumage crest reflectance under sunny sky conditions. Photon catch is approximately 13% greater for A than B. B was calculated using ocular media spectra from pea fowl, provided by Hart (see Hart, 2002). Irradiance spectra were collected at a great bowerbird bower during late morning under sunny sky conditions in NE Queensland in mid-November 2008. Mean crest reflectance spectra were calculated from scans of three birds taken at 45 deg using an Ocean Optics USB 2000 spectrophotometer and spectralon white reflectance standard. The spectral absorptance of the great bowerbird SWS1 visual pigment was multiplied by the spectral transmittance of the ocular media, irradiance spectra and crest plumage reflectance spectra and normalized to A. Pigment spectral absorptance was modeled using a mathematical template based on a λ_{max} of 404 nm (Govardovskii et al., 2000). Outer segment length was assumed to be 16 μm (Morris and Shorey, 1967) with end-on specific absorbance of 0.015 μm^{-1} (Bowmaker, 1977).

adaptation between short-wavelength plumage signals and UVS tuning of SWS1 in fairy wrens of the genus *Malurus*, which they suggest may have been influenced by predation pressure. In bowerbirds, the importance of signal UV reflectance has been investigated in greatest detail in the satin bowerbird. Overall, satin bowerbird decorations and plumage have relatively high UV reflectance compared with other Australian bowerbird species (see Endler et al., 2005); however, decoration choice tests reveal no preference for UV reflectance (Borgia, 2008) and male plumage UV reflectance is not related to mating success (Savard et al., 2009). Thus, these results do not suggest that UV signaling has been a major driver for reduced ocular filtering of UV wavelengths. Alternately, selection for reduced ocular filtration of UV wavelengths may have more to do with the benefits of increased contrast sensitivity and/or increased signal-to-noise ratio of the SWS1 *via* greater VS photon catch than with the ability to perceive shorter wavelengths (Vorobyev and Osorio, 1998).

Relative cone proportions and distribution

Differences among bowerbird species in cone proportions show no obvious association with prominent features of their visual ecology, which would be expected if they were involved in tuning. For example, there is no large difference in relative percentage of SWS1 cones between bowerbirds that occupy UV-rich habitat compared with those that live in UV-limited habitat. Also, the degree of variation in cone proportions between conspecific individuals captured from the same location is similar to the variation between species, which suggests that this level of variation may not be functionally important (see supplementary material Table S3). Theoretical visual modeling indicates that the small differences in

relative cone percentages that we observed would not have much effect on visual sensitivity (Lind and Kelber, 2009). In contrast, however, comparative studies of naturally occurring variation in retinal cone distribution among ecologically divergent bird species, including the satin bowerbird, suggest that relatively small-scale variation may reflect adaptive differences (e.g. Partridge, 1989; Hart, 2001a). Thus, we cannot definitively rule out the possibility that these differences may have a significant effect on colour sensitivity.

CONCLUSIONS

We detected no clear evidence of differential visual tuning that may be related to display colour variation among bowerbird species. In general, these data are also consistent with the broader pattern of limited variation in spectral sensitivity across birds (Hart and Hunt, 2007). This relatively constant sensitivity across most birds and their evenly spaced spectral distribution of cones suggests a generalized colour visual system that is optimized to take advantage of the full range of wavelengths that are available in most diurnal terrestrial habitats. Similar explanations have been offered to explain why Hymenoptera (Chittka, 1996; Briscoe and Chittka, 2001) and anole lizards, *Polychrotidae* (Loew et al., 2002), also show little interspecific variation in spectral sensitivity despite large variation in visual ecology. This contrasts with the aquatic environment, where spectral conditions can be much more variable among habitats and may impose strong divergent selection on visual systems, which has been demonstrated to contribute to the differential tuning that drives signal divergence and reproductive isolation. Also, several recent studies (Frentiu and Briscoe, 2008; Sabbah et al., 2010; Yuan et al., 2010; Odeen et al., 2011) suggest that signals may drive tuning, indicating that this process may not be as rare as had been previously suggested.

Overall, our results lend support to the prevailing view that interspecific variations in visual spectral tuning are uncommon among birds and not a major contributing factor to signal diversity. However, further research is needed to evaluate the potential tuning significance of the widespread interspecific variation in intraretinal distribution of cone types among birds. Additionally, there could be variation among species in post-receptoral opponency wiring that would affect chromatic contrast perception and thereby influence display colour differences (see Briscoe and Chittka, 2001; Kelber et al., 2003).

The factors that have influenced divergence of bowerbird display colouration still remain poorly understood. The results of this study and two previous studies (Borgia and Keagy, 2006; Zwiers, 2009) offer no support for sensory biases operating at the level of the eye or higher levels of visual processing to shape signal design. In the absence of differential sensory biases among species, the sensory drive model suggests that differences in habitat ambient light spectra and/or signaling background colour may drive signal divergence. Endler et al. (Endler et al., 2005) examined both of these factors in a detailed spectral analysis of colour display evolution in the bowerbird family. They reported that variation in ambient light had little effect on perceived contrast of chromatic displays, which suggests limited importance in diversification of display colouration. Furthermore, it has been suggested that the importance of variation in ambient light to signal colour diversity among birds in general has perhaps been overestimated, considering that birds possess excellent colour constancy, which largely corrects for natural spectral variation experienced under most diurnal conditions (Stoddard and Prum, 2008). With respect to display contrast with background, Endler et al. (Endler et al., 2005) conclude that there has been a phylogenetic trend of increasing contrast within the bowerbird family, which they attribute mainly to decorations. However, although bowerbirds do create high contrast signaling backgrounds that enhance the overall display conspicuousness, it is not clear what is driving divergence of display colour preference among species. Reinforcement against hybridization is a possible cause of plumage colour divergence between sympatric species, but this would not explain differences in colour among allopatric species nor suggest any role in their speciation. An alternate hypothesis pertaining to decorations is that specific objects are chosen not for their spectral characteristics *per se* but rather because those objects indicate some aspect of male quality, such as the ability of male satin bowerbirds to find blue objects (which had been rare in the environment prior to the availability of man-made materials) and defend them from competing males that steal them (see Borgia, 1985). More detailed comparative analyses are necessary to better understand the causes of divergence in display colour and colour preference in bowerbirds.

LIST OF SYMBOLS AND ABBREVIATIONS

A	alanine
C	cysteine
L	leucine
LWS	long-wavelength sensitive
MSP	microspectrophotometer
MWS	medium-wavelength sensitive
PBS	phosphate-buffered saline
RPE	retinal pigmented epithelium
S	serine
SWS	short-wavelength sensitive
T	threonine
UV	ultraviolet
UVS	ultraviolet sensitive
V	valine
VS	violet sensitive
λ_{cut}	cut-off wavelength
λ_{max}	wavelength of maximum absorbance
λ_{mid}	wavelength of half-maximum absorptance
$\lambda T_{0.5}$	wavelength of 0.5 transmittance

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