

ABSTRACT

Title of Document: EVOLUTION AND SIGNIFICANCE OF SEXUAL DISPLAYS IN BOWERBIRDS (PTILONORHYNCHIDAE)

Brian James Coyle, Biology PhD, 2013

Directed By: Professor, Gerald Borgia PhD, Department of Biology

The spectacular diversity of sexual displays among taxa is shaped by many factors including female choice, habitat, ecology and learning. Bowerbirds, Ptilonorhynchidae, have many display elements that are highly differentiated among species providing an excellent system for studying processes of display evolution. This dissertation examines several aspects of display evolution including co-evolution of visual spectral sensitivity and color displays in the context of sensory drive theory, interspecific learning of behavioral displays and hybridization, and the significance of collected objects used in display. Physiological and molecular genetic investigation of peripheral visual systems revealed no species difference in spectral sensitivity that would explain the large interspecific differences in male and female display color preferences. Results did reveal that bowerbirds are sensitive to relatively short wavelength ultraviolet light which suggests that they may use this waveband in sexual communication. Male great bowerbirds (*Chlamydera nuchalis*) appear to arrange collected objects in a size specific pattern but there is no evidence for the recently proposed hypothesis that this pattern is designed to create a visual illusion for females. The purpose of the size related arrangement of objects remains unclear. Spotted (*Chlamydera maculata*) and great bowerbirds (*C. nuchalis*) are highly differentiated in display, size and appearance yet there

is a high frequency of bidirectional hybridization in a contact zone in northwest Queensland, Australia. Evidence indicates that males in this contact zone learn displays from the other species, which could influence hybridization, as in other avian hybrid systems.

EVOLUTION AND FUNCTIONAL SIGNIFICANCE OF SEXUAL DISPLAYS
IN BOWERBIRDS (*Ptilonorhynchidae*)

By

Brian James Coyle

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Advisory Committee:
Professor Gerald Borgia, Chair
Professor Marjorie Reaka
Associate Professor Karen Carleton
Associate Professor Al Uy
Associate Professor David Hawthorne

Preface

This dissertation includes three chapters that each contains an abstract, introduction, methods, results, and discussion section. Tables, figure captions and figures are presented at the end of the discussion section. The first chapter is presented in its published form (*The Journal of Experimental Biology*. 2012. 215, 109-1105), the second chapter has been accepted for publication pending revisions and is presented in its submitted form (*Ecology and Evolution*. 2012), and the third chapter is presented as a manuscript. The dissertation concludes with a single bibliography section that includes all references cited for the three chapters.

Dedication

This dissertation is dedicated to my mother, Carol L. Coyle, and late father, James A. Coyle, whose love, encouragement and example have been an invaluable source of support and to my children, Eamon and Aide, who give me great joy and inspire me to achieve.

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Table of Contents

Preface.....	ii
Dedication.....	iii
Acknowledgements.....	iv
Table of Contents.....	v
List of Tables.....	vi
List of Figures.....	vii
Introduction	1
Chapter 1: Limited variation in visual sensitivity among bowerbird species suggests that there is no link between spectral tuning and variation in display coloration.....	7
Abstract.....	7
Introduction.....	8
Methods.....	16
Results.....	23
Discussion.....	27
Tables.....	37
Figure Captions.....	48
Figure.....	52
Chapter 2: Does learning of display traits influence frequent hybridization between two species of bowerbirds with distinctive sexual display?.....	63
Abstract.....	63
Introduction.....	64
Methods.....	67
Results.....	71
Discussion.....	74
Table.....	81
Figure Captions.....	88
Figures.....	90
Chapter 3: No evidence that great, spotted or western bowerbirds create visual illusions to attract females and increase mating success.....	93
Abstract.....	93
Introduction.....	94
Methods.....	97
Results.....	99
Discussion.....	100
Tables.....	104
Figure Captions.....	105
Figures.....	106
Bibliography.....	109

List of Tables

Chapter 1

1. Species habitats, display colors, and sampling	37
2. Amino acid residues at spectrally important loci	38
3. Variation in opsin sequence	39
4. Spectral characteristics of visual pigments	40
5. Spectral characteristics of visual oil droplets	42
S1. Opsin sequencing primers	44
S2. Accession numbers	45
S3. Relative cone distribution	46

Chapter 2

1. Sampling locations and data types collected	81
2. Plumage scoring index	82
3. Morphological measurements	83
4. Bowers used in study	84
5. Principal Component factor loadings, eigenvalues, % variance	85
6. Behavioral displays	86
7. Bower construction	87

Chapter 3

1. Example photos of bower decorations	107
2. Illustrations of object distributions on bowers	108

List of Figures

Chapter 1

1. Oil droplet microimages	52
2. Visual pigment pre and post bleaching spectra	53
3. Visual pigment difference spectra	54
4. Oil droplet absorbance spectra	55
5. Oil droplet cut-off wavelength spectra	57
6. Ocular media transmittance spectra	59
7. Cone proportions	60
8. Calculated photon catch for satin bowerbird visual pigments	61
9. Calculated visual sensitivity of great bowerbirds	62

Chapter 2

1. Maps of study locations	90
2. Pictures of hybrid, spotted and great bowerbird and bowers	91
3. Hybrid detection - Principal Components Analysis	92

Chapter 3

1. Example pictures of bower decorations	107
2. Illustrations of possible object distributions	108

Introduction

There is spectacular diversity of sexual displays among taxa. Charles Darwin was first to suggest that these traits have arisen through sexual selection (Darwin 1871). The theory of sexual selection addresses competition for mates that occurs within and between sexes (i.e. mate choice) and leads to variation in individual reproductive success. Darwin's insights into display evolution and mate choice were largely rejected by Alfred Russell Wallace (1878), who independently conceived of Natural Selection, and were controversial for decades but are now a cornerstone of evolutionary theory. The important role of sexual displays in mate choice has been demonstrated by extensive research in many species (e.g. Andersson 1994).

Although the importance of sexual displays is well established, display evolution remains a controversial topic and active area of research. Multiple hypotheses have been offered to explain the evolution of sexual displays. The "good genes" hypothesis suggests displays communicate reliable information about male quality that allows females to make informed comparisons between potential mates and choose the best one (e.g. Fisher, 1915; Hamilton and Zuk, 1982; Maynard Smith, 1976). These indicators of male quality may become very costly to produce and maintain and expose the bearer to considerable risk. The "handicap" hypothesis predicts that the inherent cost of certain displays and the variation among males in their ability to bear those costs ensure that the traits are honest indicators of relative male quality (Zahavi 1975, 1977). "Runaway selection" is a competing hypothesis that suggests displays evolve through a non-adaptive

process that, in most models, is sustained by genetic correlation between display and preference (Fisher, 1930; Lande, 1981, 1987). Display elaboration via run-away is driven by open-ended preference and limited by natural selection. Sensory drive is a theory of signal evolution that addresses display design or form, including characteristics such as color, pattern, and frequency (e.g. Lythgoe, 1979; Endler, 1992a,b; Endler and Basolo, 1998). Sensory drive shapes displays to maximize communication efficiency within the particular constraints of local habitat parameters and sensitivities of the sensory system that receive the display. Thus, differences in display design are largely attributed to variation in habitats and sensory abilities among taxa. For example, display color differences among bird species may be due to the variation in habitat light spectral composition and differences in their visual spectral sensitivity (see Hill and McGraw, 2006). A variation of sensory drive called sensory exploitation suggests that males may evolve displays that take advantage of sensory biases which have evolved in other behavioral contexts, such as foraging, to manipulate females into mating (Ryan and Rand, 1990; Kirkpatrick and Ryan, 1991; Endler and Basolo, 1995). Sensory exploitation may be mal-adaptive for females if the display is not somehow correlated with male quality and may therefore lead to selection on females to eliminate or avoid susceptibility to the display.

In this dissertation, I explore the evolution and development of sexual displays in species of bowerbirds, family Ptilonorhynchidae. Most bowerbirds are polygynous species with lek-like mating systems. They have long been an important model for studying sexual selection (e.g. Darwin, 1971; Marshall, 1954; Gilliard, 1969; Borgia,

1985; Frith et al, 2004). Females are highly selective in choosing mates and mating success is strongly skewed among males (e.g. Borgia, 1985; Borgia 1992). Males display in various modalities and express morphological, behavioral and off-body elements. They build a courtship structure called a bower that provides protection to females from forced copulation as they attend to behavioral display. Males decorate their bower and the surrounding display court with objects of various type and color that they collect from their environment and arrange in specific patterns (e.g. Borgia, 1985; Diamond, 1987; Lenz, 1994; Madden et al. 2004; Endler et al., 2005; Endler and Day, 2006; Borgia, 2008). They also destroy bowers of other males and plunder decorations (e.g. Borgia, 1985b; Borgia and Mueller, 1992). Courtship consists of vocalizations, behavioral elements and displays of their colorful plumage. All display types are highly differentiated throughout the bowerbird family and studies in multiple species reveal a strong correlation between mating success and many display elements (e.g. Borgia, 1985; Borgia, 1992; Madden, 2003; Frith et al. 2004).

Display development in bowerbirds is complex and is thought to involve an extensive learning component (e.g. Marshall, 1954; Borgia, 1986; Loffredo and Borgia, 1986; Diamond, 1988; Frith et al., 2004; Madden, 2008; Endler et al., 2010). Bowerbirds are long lived species and males are not fully mature until more than five years old. During their juvenile period they observe the displays of adult males and practice display with other juveniles. Many species are also excellent vocal mimics demonstrating that learning is involved at least in vocal display acquisition (e.g. Loffredo and Borgia, 1986; Coleman et al., 2007; Kelly and Healy, 2010).

In chapter one, I test the sensory drive hypothesis in bowerbirds by investigating the relationship between visual spectral sensitivity and the color of plumage and decoration displays. Bowerbirds have strong and specific color preferences and dislikes that differ between species. Sensory drive suggests that these differences may be driven by variation in spectral sensitivity that has arisen as a result of local adaptation to habitat (Endler 1992b). Spectrophotometry and microspectrophotometry were used to measure spectral transmission through ocular media (lens, cornea, and aqueous humor) and measure sensitivity of retinal photoreceptors that include four spectrally distinct cone receptors involved in color vision. I also used fluorescent microscopy to quantify the relative number of cone types from mounted retinal tissue. Furthermore, I sequenced the genes that code for opsin pigments to estimate photoreceptor spectral sensitivity. I found no differences in spectral sensitivity among 13 species that would explain the large interspecific variation in display coloration. However, I did find that bowerbird's lenses are relatively transparent to ultraviolet (UV) wavelengths which could influence the evolution of UV reflective displays.

In chapter two, I investigated the occurrence of natural hybridization between two bowerbird species, *Chlamydera nuchalis* and *C. maculata*. In other sympatric species of bowerbirds hybridization is rare or absent which may be attributable to prezygotic reproductive isolation that is due to large differences in appearance and display (see Coyne and Orr, 2004). In this study I detected over 20% hybrid individuals based on phenotype analysis. I also provide evidence of mitochondrial introgression and show that

females of both species hybridize. Given that females show a high degree of effort in intraspecific mate choice (Borgia, 1995a; Madden, 2003) it is not clear why they would mate with males of another species. Based on the distribution of bowers throughout the contact zone, it does not appear that females lack access to males of their own species, which is one of the more common reasons that bird species hybridize. Studies of other avian hybrid systems show that display learning between species drives hybridization. I provide behavioral evidence that male bowerbirds in this contact zone may learn displays from the other species.

In chapter three I investigate hypotheses about the design of decoration displays. Recent work suggests that great bowerbirds, *Chlamydera nuchalis*, arrange objects to create a visual illusion called Forced Perspective that attracts females and increases male mating success (Endler et al. 2010; Kelly and Endler, 2012a,b). According to this hypothesis males arrange objects from small to large with distance from the bower to create an even size gradient such that all objects subtend the same visual angle to the female eye. The supposed effect is to make all objects appear the same size and make displays in the foreground appear closer. However, the data from these studies does not support the claim that males do create even size gradients nor does it show a link between the supposed illusion and mating success (Anderson, 2012; Borgia et al., 2012). I hypothesize that males are simply placing smaller objects closer to the bower for the practical purpose of avoiding interference from obstacles during courtship display. I conducted a survey of human subjects who assessed patterns of size-related bower decoration design. Survey participants were instructed to evaluate the size related pattern

of object distribution in decoration displays based on visual examination of digital images. I included 40 bowers from great (*C. nuchalis*) spotted (*C. maculata*) and western (*C. gutatta*) bowerbirds, which share similar decoration schemes. Results of this study do not support either hypothesis, however I suggest that the obstacle avoidance hypothesis may be challenging for naive observers to detect.

Chapter 1: Limited variation in visual sensitivity among bowerbird species suggests that there is no link between spectral tuning and variation in display colouration

ABSTRACT

Variation in visual spectral tuning has evolved in concert with signal color 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 color 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 colored 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-wavelengthsensitive-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 color. 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.

INTRODUCTION

Darwin (e.g. 1871) and 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 like 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 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 detailed review of avian visual system see Hart, 2001b). All birds studied to date have been shown to utilize the vitamin A₁-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 very short wavelength sensitive (SWS1), short wavelength sensitive (SWS2), rhodopsin like (Rh2) and medium/long wavelength sensitive (M/LWS) classes and have wavelengths of maximum absorbance (λ_{\max}) between ~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; 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 effect 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 a medium-wavelength-sensitive 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 (i) occupy different light habitats that might tune their visual sensitivity differently, (ii) experience strong selection on colour communication, and (iii) are highly differentiated in signal colour. This approach has the advantage of potentially revealing if 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, the 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., 2004). Bowerbirds have been an important model for studying mate choice and display trait evolution, with much of this research being 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; e.g. 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, 1995b, a; 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., 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 (2005) analyzed plumage and decoration reflectance spectra and argued that especially large differences in plumage colour between sympatric bowerbirds compared to allopatric pairs suggest a process of “reinforcement” for species recognition (Dobzhansky, 1937). However, the accompanying evidence required to demonstrate this process, such as intra-specific character displacement from without 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 (2006) because it used coloured grapes (a popular food item for bowerbirds but never used as a decoration) both as food and display objects making it difficult to interpret their experiment. In contrast, 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 (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 of 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 (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 sequence 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 Rh1 opsin in these six species to more completely characterize their full opsin complement. The data collected in this study represents the

most extensive comparison of visual sensitivity within an avian family that has been performed to date, and 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 that bowerbirds have, to assess whether tuning has occurred and may be related to bowerbird display divergence, and to 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.

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 clade (Kusmierski et al., 1993) (Table 1)

Collection and sacrifice

Birds were captured by cage trap or mist net in New South Wales or Queensland Australia during November and December of 2008. Collecting large numbers of bowerbirds is discouraged by wildlife authorities and, 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 five 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 one hour and then humanely sacrificed 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 Quiagen DNeasy and RNeasy kits, 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).

Degenerate PCR primers were developed for each opsin (Primer 3, Rozen and Skaletsky, 2000) based on consensus sequence 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 Table 1 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; SWS2 (46, 49, 52, 91, 93, 94, 116, 122, 164, 207, 261, 269, 292); Rh2 (122, 222, 295); M/LWS (164, 181, 261, 269, 292) (see Yokoyama, 2002; Takahashi and Ebrey, 2003; Hunt et al., 2009). Amino acid position numbering for opsins correspond 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 using either Taq (Invitrogen) or Dynazyme (NEB) polymerase and following manufacturer protocols, optimized when necessary. PCR products were purified using Quiaquick kits (Quiagen) and then cycle-sequenced using primers and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sanger sequencing was performed on either an ABI 3730xl or 3100 DNA Analyzer (Applied Biosystems). All gene fragments were sequenced in both directions.

Chromatographs were imported into Sequencher version 5.0 (Gene Codes Corp.) 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, 2004; Hart et al., 2011). Briefly, dark-adapted eyes were dissected under infra-red illumination with the aid of an image converter in cold (4°C) phosphate-buffered saline (PBS: 340 mOsmol kg⁻¹, pH 7.2; Oxoid Pty. Ltd., Australia). Small (1–2 mm²) 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 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 $1 \times 5 \mu\text{m}$ for cone outer segments, $1 \times 10 \mu\text{m}$ for rod outer segments and $1 \times 1 \mu\text{m}$ 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 prebleach spectrum. Each outer segment was then bleached with white light for 1–2 min and subsequent sample and baseline scans made to create a postbleach spectrum (and thus confirm that any putative visual pigments were photolabile). Scans of oil droplets were converted from transmittance to absorbance 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 prebleach absorbance spectra were analysed as described elsewhere (Hart, 2002) following the methods of MacNichol (1986) and 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 prebleach absorbance spectrum was calculated by averaging acceptable individual (non-normalized) absorbance spectra and overlaid with a vitamin-A1-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 postbleach spectrum from the prebleach spectrum, and analysed and

averaged in the same way as the prebleach spectra. Microspectrophotometry of avian photoreceptors is challenging due to 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 absorbance spectra were.

Oil droplet absorbance spectra were normalized to the maximum and long-wavelength offset absorbances 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 absorbance 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 absorbance by the line tangent to the oil droplet absorbance curve at half maximum measured absorbance (Lipetz, 1984). For comparison with other studies, the wavelength corresponding to half maximum measured absorbance (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, PX-2 pulsed xenon lamp (spectral output of 220-750 nm), and Spectrasuite data acquisition software (Ocean Optics, 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, AD; anterior-ventral, AV; posterior-dorsal, PD; and posterior-ventral, PV) using the pecten as a marker for orientation (Hart, 2001a) and the tissue left in cold (4°C) PBS (340 mOsmol kg⁻¹) for 1h to promote separation of the neural retina from the retinal pigmented epithelium (RPE). The tissue was then fixed for 2 minutes in 4% paraformaldehyde in 0.1M 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 Figure 1). Epifluorescent microscopy was used to discriminate between C- and T-type droplets, since C-type oil droplets fluoresce under ultraviolet 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.

RESULTS

Opsin sequence

Opsin cDNAs including all seven transmembrane domains (TM) were sequenced for six species (see supplementary Table 2 for sequence accession numbers). The average lengths of the amino acid sequences translated from cDNA are 315 aa for SWS2, 285 aa for Rh2, 334 aa for LWS, and 324 aa for Rh1. gDNA sequence for LWS and Rh2 opsin genes was obtained from 12 species and contains most exonic sequence including all of TM 1, 2, and 4-7, partial TM 3, and all key amino acid sites listed above. Relatively

limited gDNA sequence was obtained for the SWS2 despite efforts to optimize primer design and PCR conditions, however successfully amplified portions include TM5-7 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 opsin that were 270 and 222 aa long, respectively.

Comparison of translated opsin amino acid sequences revealed little variation between bowerbirds (Table 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. Absorbance measurements could not be 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 to 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 (Tables 4-5). They possess a single class of rod photoreceptor that has a medium-wavelength-sensitive visual pigment with a mean λ_{\max} between 501–506 nm and four subtypes of single cone photoreceptor that are maximally sensitive to either violet- (VS), short- (SWS), medium- (MWS) or long-wavelengths (LWS); and also long-wavelength-sensitive double cones, both the principal and accessory members of which contain the same LWS visual pigment as the LWS single cones (Figures 2-3 show absorbance and difference spectra from visual pigments in satin bowerbird). Mean λ_{\max} values for the VS, SWS, MWS and LWS visual pigments across the six species of bowerbird studied were between: 404–410 nm, 454 nm, 503–511 nm and 558–568 nm, respectively.

VS single cones contained a transparent ‘T-type’ oil droplet that had negligible absorbance (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 absorbance characteristics (Figures 4-5; Table 5). 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 nm, 508–515 nm, 558–571 nm and 412–425 nm, respectively; whereas in the ventral retina they were 424–441 nm, 516–523 nm, 566–573 nm 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 absorbance of the A-type oil droplets was very low (0.05–0.08), but in the other four species the mean peak absorbance was considerably greater (0.13–0.38) and λ_{cut} values ranged from 456–492 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 similar spectral absorbance characteristics to 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 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 (Figure 6). Below 400 nm, the transmittance dropped rapidly and wavelengths below about 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 would be required to test this properly (Figure 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 table 3 for cone count data by quadrant for six species).

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 transmits significantly more UV wavelengths than 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 that indicates spectral tuning has probably not been a significant cause of, or response to, species divergence in display colour.

Visual pigments

We show that visual pigments are spectrally similar among bowerbirds based on comparison of spectral absorbance 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 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 review of opsin tuning sites and spectral significance of various amino acids see Yokoyama, 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 and Frith, 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 to the dorsal retina in most species. The difference is 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 intra-retinal variation has also been observed in other species, which suggests it may be common across many bird taxa (e.g. Hart, 2004; Hart et al., 2006). Experimental evidence suggests 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 to cones in the dorsal retina (Hart et al., 2006). Due to 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 (Figure 8).

In contrast to the typical pattern of intra-retinal variation, however, the λ_{cut} values of C-type droplets did not differ between the ventral and dorsal SWS2 cones in great and toothbilled bowerbirds. Rather, the C-type droplet λ_{cut} values in these two species were similar to the dorsal SWS2 cones of all species (i.e. about 12 nm shorter wavelength than in ventral cones). Consequently, ventral SWS2 cones may have substantially greater photon catch in great and toothbilled bowerbird compared to 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 habitat 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 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 while most species with VS pigments have $\lambda T_{0.5} \geq 359$ nm. Hastad et al. (2009) calculated that a shift from $\lambda T_{0.5}$ 365 nm to 338 nm increases SWS1 photon catch by about 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 almost no effect on SWS1 photon catch. Based on these calculations, they hypothesized that a loss of UV filtering ocular pigment likely preceded and promoted 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 340-352 nm) suggest the possibility that they represent the proposed transitional link from a VS to 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 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 other birds with a similar VS visual pigment (Figure 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 by simulating their

visual performance using optical parameters of a typical VS type system (i.e. $\sim \lambda T_{0.5} 362$ nm). Furthermore, the greater UV sensitivity that we detected would likely enhance perceived colour contrast of the crest, since males typically display their crest during courtship against a low UV reflecting background (for characterization of spectral conditions see 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 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. (2011) provide evidence for possible co-adaptation between short wavelength plumage signals and UVS tuning of SWS1 in *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 to 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 versus those that live in UV limited habitat. Also, the degree of variation in cone proportions between con-specific 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 Table 3). 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 anoles 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 differential tuning that drives signal divergence and reproductive isolation. Also, several recent studies suggest signals may drive tuning, indicating 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 intra-retinal 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 (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 that 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 (2005) conclude that there has been a phylogenetic trend of increasing contrast within the bowerbird family, which they attribute mainly to decorations. However, while 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.

Abbreviations and symbols used

A alanine

C cysteine

L leucine

LWS long wavelength sensitive

MSP microspectrophotometer

MWS medium wavelength sensitive

PBS phosphate-buffered saline

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

$\lambda_{T_{0.5}}$ wavelength of 0.5 transmittance

TABLES

Table 1. Species habitat, predominant display colours, and sampling information. Habitat type: rain forest (RF), forest edge (FE), open woodland and scrubland (OW), montane forest (MF), cloud forest (CF). Clade: monogamous ancestor (Anc) does not clear court, build bower, or decorate, avenue bower builders (A), maypole bower builders (M). Display colours columns indicate the most prominent plumage and decorations colours. Plumage colours are constant across species but there is considerable geographic variation in decoration colours used by some species, e.g. Vogelkop. MSP data may include oil droplet absorbance and visual pigment absorbance.

Species name	# birds & sex	Habitat type	Clade	Display colours		MSP	Oc med	cDNA	gDNA
				Plumage	Decoration				
Green catbird (<i>Ailuroedus crassirostris</i>)	1M	RF	Anc	Green	None	✓	✓	✓	
Tooth-billed (<i>Scenopooetes dentiostriis</i>)	1M	RF	M	Olive brown	Green leaves	✓		✓	✓
Golden (<i>Prionodura newtoniana</i>)	1M	RF,RE	M	Yellow	Green				✓
Streaked (<i>Amblyornis subalaris</i>)	1M	MF	M	Orange/ red	Blue, Purple, Reds				✓
Macgregor (<i>Amblyornis macgregoriae</i>)	1M	MF	M	Orange/ red	Red, Orange, Yellow				✓
Archbold (<i>Archboldia papuensis</i>)	1M	MF	M	Orange/ yellow	Blue, Green				✓
Vogelkop (<i>Amblyornis inornatus</i>)	1M	CF	M	Olive brown	Blue, Orange, Red				✓
Regent (<i>Sericulus chrysocephalus</i>)	2F	RF	A	Yellow Black	Blue, Yellow	✓	✓	✓	✓
Satin (<i>Ptilonorhynchus violaceus</i>)	1M, 1F	RF, FE	A	Blue/black	Blue, Yellow	✓	✓	✓	✓
Fawn-breasted (<i>Chlamydera cerviniventris</i>)	1M	OW,FE	A	Dark Cinnamon	Green				✓
Great (<i>Chlamydera nuchalis</i>)	2M	OW	A	Pink/lilac	Green, Red, Blue	✓	✓	✓	✓
Spotted (<i>Chlamydera maculate</i>)	1M	OW	A	Pink/lilac	Green, Yellow, Red, Blue	✓	✓	✓	✓
Western (<i>Chlamydera guttata</i>)	1M	OW	A	Pink/lilac	Green, Red, Blue				✓

Table 2. Comparison of opsin amino acid residues between species at spectrally important loci. Comparison of visual pigment opsin amino acid residues at known spectral tuning sites and visual pigment spectral absorbance characteristics measured using microspectrophotometry. Species are compared to consensus sequence for tuning sites with a dot indicating total agreement with consensus. Residues that differ from consensus are highlighted in grey. All tuning sites for violet (SWS1), green (Rh2), and red (LWS) opsin were sequenced for all species. Rod (Rh1) and blue (SWS2) opsin tuning sites were sequenced in full only for six species for which retinal tissue was available. Additionally, three blue opsin tuning sites were sequenced for the remaining seven species (*). SWS1 opsin data for positions 86, 90 and 93 comes from Zwiers (2009). λ_{max} is the mean wavelength (nm) of maximum absorbance of the visual pigment. Rod opsin was not sequenced for seven species. Amino acid numbering follows that of bovine rod opsin.

Visual pigments and corresponding opsins	SWS1 λ_{max}	Violet	SWS2 λ_{max}	Blue	Rh2 λ_{max}	Green	LWS λ_{max}	Red	Rh1 λ_{max}	Rod
Consensus residues		86C, 90S, 93T		46L, 49A, 52V, 91S, 93T, 94A, 116A, 122F, 164G, 207L, 261F, 269S, 292S		122Q, 222S, 295S		164S,181H, 261Y, 269T, 292A		122E, 222C, 295A
<i>A. crassirostris</i>	406	.		52L	507	.	562	.	501	.
<i>S. dentirostris</i>		.		52L		.	563	.	506	.
<i>S. chrysocephalus</i>	408	.		.		.	563	.	503	.
<i>P. violaceus</i>	410	.	454	.	511	.	562	.	503	.
<i>C. nuchalis</i>	404	.		.	503	.	564	.	503	.
<i>C. maculata</i>		505	.
<i>C. cerviniventris</i>		.		*261F, 269S, 292S		.		.		N/A
<i>C. gutatta</i>			N/A
<i>A. macgregoriae</i>			N/A
<i>A. subalaris</i>			N/A
<i>A. papuensis</i>			N/A
<i>A. inornatus</i>			N/A
<i>P. newtonia</i>			N/A

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

Opsin	SWS2								RH2		LWS			RH1	
	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
<i>A. crassirostris</i>	T	L	I	I	I	T	D	E	V	V	I	V	.	.	S
<i>S. dentiostriis</i>	T	L	I	.	I	.	D		V	.	.	V	.	V	S
<i>S. chrysocephalus</i>
<i>P. violaceus</i>
<i>C. nuchalis</i>	L	.	.
<i>C. maculata</i>	I
<i>C. cerviniventris</i>							
<i>C. gutatta</i>							
<i>A. macgregoriae</i>								.	.	V	.	.	V	.	.
<i>A. subalaris</i>								.	.	V	.	.	V	.	.
<i>A. papuensis</i>								.	.	V	.	.	V	.	.
<i>A. inornatus</i>								.	.	V	.	.	V	.	.
<i>P. newtonia</i>								.	.	V	.	.	V	.	.

Table 4. Characteristics of visual pigments measured via microspectrophotometry

Visual Pigments	Single Cones				Double Cones		Rods
	VS	SWS	MWS	LWS	Principal	Accessory	
<i>Satin – Ptilonorhynchus violaceus</i>							
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
<i>N</i>	3	4	2	3	4	5	11
<i>Great – Chlamydera nuchalis</i>							
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	565.8	562.6	572.6	505.7
<i>N</i>	1	-	2	1	10	2	4
<i>Catbird – Ailuroedus crassirostris</i>							
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	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	570.4	560.8	570.1	505.0
<i>N</i>	1	-	2	1	3	1	3
<i>Regent – Sericulus chrysocephalus</i>							
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
<i>N</i>	1	-	-	-	11	4	9
<i>Toothbilled – Scenopoetes dentirostris</i>							
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
<i>N</i>	-	-	-	1	3	1	3
<i>Spotted – Chlamydera maculata</i>							
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 \pm 1.9
λ_{max} of mean difference spectrum (nm)	-	-	-	-	-	-	504.7
N	-	-	-	-	-	-	2
<i>Values are \pm 1 standard deviation</i>							

Table 5. Characteristics of coloured oil droplets measured via microspectrophotometry

Oil droplets	Single cones								Double cones			
	T-type	C-type		Y-type		R-type		P-type		A-type		
		D	V	D	V	D	V	D	V	D	V	
<i>Satin – Ptilonorhynchus violaceus</i>												
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	
<i>Great – Chlamydera nuchalis</i>												
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	
<i>Green catbird – Ailuroedus crassirostris</i>												
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	
<i>Regent – Sericulus chrysocephalus</i>												
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	

<i>N</i>	8	10	6	11	10	10	10	21	11	3	4
Toothbilled – <i>Scenopoetes dentirostris</i>											
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
<i>N</i>	6	10	11	10	10	10	10	13	10	1	1
Spotted – <i>Chlamydera maculata</i>											
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 ± .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 ± 0.0
<i>N</i>	4	9	10	10	10	10	10	10	10	-	-
<i>Values are ± 1 standard deviation</i>											

Table S1. Primers for LWS (RDO), Rh2 (GRO), SWS2 (BLO) and Rh1 (RH1) opsins. Primer names are preceded with an F for forward and R for reverse and end with the number of the exon in which they are located. Various primer combinations were sometimes used within each opsin

Forward primers		Reverse primers	
Name	Sequence (5' to 3')	Name	Sequence (5' to 3')
FRDO1	GACACGACGACGAGGACAC	RRDO2	ATCAGCGTGGTCAACCAGA
FRDO2	GCTGGGTGTACAACCTGACG	RRDO3	TCTCCTGGATCTGGTCCTG
FRDO3	GTGGTCTGCAAACCCTTCG	RRDO4	TCTGCTACCTGCAAGTCTGG
FRDO4	GTCCTACATGGTGGTGCTGA	RRDO5	CCAAGCGCGCCACCATCTA
FRDO5	AGAAGGCGGAGAAGGAGGT	RRDO6	GTGTCCAACCTCCTCCGTGTC
FGRO1	TCGAGTACCCGCAGTACCT	RGRO2	CATCGCTTTCACCTGGGT
FGRO2	CATCGAGCGCTACATTGTCA	RGRO3	CGTCATCATCTTCTTCTCCTAC
FGRO3	ACAACCCCGACTTCCACA	RGRO4	CTCCAAGAGCTCCTCCCTCT
FGRO4	AGAAGGCGGAGAAGGAGGT	RGRO5	GCATGATCACCAACAATCTGC
FBLO1	GCTGAGCCCCTTCCTGGT	RBLO2	CTGGATCTTCGGCCTCATCG
FBLO2	GGTTCCTGGTCATCTGCAAG	RBLO3	CGTCATCGTCTTCTCCTACGG
FBLO3	GACTGGTACACGACGGACAA	RBLO4	GTCTTCTCCAAGGCCTCCAC
FBLO4	GATGGTGGTGGTGATGGT	RBLO5	AGGTGTCCTCGGTGTCCTC
FRH101	AACCATGAACGGGACAGAAG	RRH102	GTGTTGCCTTCTCCTGGATC
FRH102	CACTGGTGGTCTGGCTATC	RRH103	CTTCTGCTATGGGAACCTGGT
FRH103	TCTGAAGCCAGAGGTCAACA	RRH104	CCATCTTCATGACCATCCC
FRH104	AAGTGACTIONGATGGTCATC	RRH105	CAAGACAGAGACCTCCTCCG

Table S2. Accession numbers for cDNA (black) and gDNA (grey) sequences

Species		Opsin			
Common name	Scientific name	SWS2	Rh2	LWS	Rh1
Green catbird	<i>A. crassirostris</i>	JQ034386	JQ034374	JQ034369	JQ034381
Tooth-billed	<i>S. dentiostriis</i>	JQ034387, JQ218125	JQ034375, JQ034398	JQ034368, JQ218137	JQ034380
Golden	<i>P. newtoniana</i>	JQ218126	JQ034399	JQ218138	
Streaked	<i>A. subalaris</i>	JQ218127	JQ034400	JQ218139	
Macgregor	<i>A. macgregoriae</i>	JQ218129	JQ034402	JQ218141	
Archbold	<i>A. papuensis</i>	JQ218130	JQ034403	JQ218142	
Vogelkop	<i>A. inornatus</i>	JQ218128	JQ034401	JQ218140	
Regent	<i>S. chrysocephalus</i>	JQ034389, JQ218124	JQ034376, JQ034397	JQ034370, JQ218136	
Satin	<i>P. violaceus</i>	JQ034388, JQ218119	JQ034377, JQ034392	JQ034371, JQ218131	JQ034383
Fawn-breasted	<i>C. cerviniventris</i>	JQ218123	JQ034396	JQ218135	
Great	<i>C. nuchalis</i>	JQ034391, JQ218122	JQ034378, JQ034395	JQ034372, JQ218134	JQ034384
Spotted	<i>C. maculata</i>	JQ034390, JQ218121	JQ034379, JQ034394	JQ034373, JQ218133	JQ034385
Western	<i>C. guttata</i>	JQ218120	JQ034393	JQ218132	

Table S3. Mean cone percentage for different retinal quadrants. Mean % for total eye only calculated when data for all four quadrants was available. Some quadrants could not be counted because retinal pigmented epithelium did not separate from neural retina. Quadrants are anterior dorsal (AD), anterior ventral (AV), posterior dorsal (PD), and posterior ventral (PV). Oil droplet types are indicated by T, C, Y, R, P and refer to the oil droplets found in the four single cone types: SWS1, SWS2, MWS, and LWS and the principal member of the LWS double cones, respectively.

Species	Retinal area	Field of view (n)	Oil droplet type (%)				
			T	C	Y	R	P
Green catbird <i>A. crassirostris</i>	AD	8	9.1	15.3	22.5	15.9	37.2
	AV	6	9	14.8	22.7	18.7	34.8
	PD	8	8.1	16.3	22.2	17.1	36.2
	PV	10	6.4	12.3	17.6	16.2	47.5
	Mean		8.2	14.7	21.2	17	38.9
Toothbilled <i>S. denti-rostris</i>	AD	7	3.9	15	19.4	20.1	41.5
	AV	5	9	17	22.6	18	33.5
	PD	7	8.4	18.6	21	17	34.9
	PV	10	4.9	11.7	17.4	16.7	49.3
	Mean		6.5	15.6	20.1	18	39.8
Regent <i>S. chrysocephalus</i>	AD	2	8.2	7.7	17.2	23.1	43.7
	AV	9	4.4	9.7	23	23.7	39.4
	PD	4	5.1	11.9	20.5	20.5	41.9
	PV	6	4.7	9	22.4	20.8	43.1
	Mean		5.6	9.6	20.8	22	42
Great <i>C. nuchalis</i> 1	AD	10	6.3	8.8	15.1	18.5	51.3
	AV	8	8	13.3	19.7	20.5	38.6
	PD	-	-	-	-	-	-
	PV	5	5.1	10.5	16	19.5	49
Great <i>C. nuchalis</i> 2	AD	-	-	-	-	-	-
	AV	10	7.7	14.1	21.3	18.9	38.1
	PD	5	5.3	12.9	18.8	15	48
	PV	-	-	-	-	-	-
Great <i>C. nuchalis</i> 1 & 2 combined	AD	10	6.3	8.8	15.1	18.6	51.2
	AV	18	7.9	13.6	20.4	19.7	38.3
	PD	5	5.4	12.8	18.8	15	48
	PV	5	5.1	10.5	16	19.5	49
	Mean		6.2	11.4	17.6	18.2	46.6
Satin <i>P. violaceus</i> 1	AD	9	5.1	12.7	17.2	20.7	44.4
	AV	-	-	-	-	-	-
	PD	7	7.8	12.4	21.3	22.1	36.4
	PV	-	-	-	-	-	-
Satin <i>P. violaceus</i> 2	AD	8	9	12.2	21	18	39.9
	AV	4	4.3	8.5	16.4	22	48.8
	PD	-	-	-	-	-	-
	PV	9	6.2	10.8	17.5	18.1	47.4
Satin <i>P. violaceus</i> 1 & 2 combined	AD	17	7.6	12.3	19.6	19	41.4
	AV	4	4.3	8.5	16.4	22	48.8
	PD	7	7.8	12.4	21.3	22.1	36.4
	PV	9	6.2	10.8	17.5	18.1	47.4
	Mean		6.5	11	18.7	20.3	43.5

Spotted <i>C. maculata</i>	AD	-	-	-	-	-	-
	AV	-	-	-	-	-	-
	PD	9	11.6	15	17.7	17.8	38
	PV	-	-	-	-	-	-

FIGURE CAPTIONS

Figure 1: Photomicrographs of satin bowerbird (*Ptilonorhynchus violaceus*) retinal tissue at X1000 showing each class of colored oil droplets. T, C, Y, R and P correspond to 'transparent' oil droplets in the inner segment of short-wavelength-sensitive 1 (SWS1) cones, 'colorless' 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 .

Figure 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 overlaid 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 include measurements from LWS single cones and both the principal and accessory members of double cones. SWS, short-wavelength-sensitive; VS, violet sensitive.

Figure 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.

Figure 4: Mean absorbance 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.

Figure 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.

Figure 6: Ocular media transmittance spectra for five species. The wavelengths of 0.5 normalized transmittance ($\lambda_{T0.5}$) of the ocular media of the green catbird (*Ailuroedus crassirostris*), and satin (*Ptilonorhynchus violaceus*), regent (*Sericulus chrysocephalus*), great (*Chlamydera nuchalis*) and spotted bowerbird (*Chlamydera maculata*) were 340, 343, 349, 349 and 352 nm, respectively.

Figure 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 (*Ptilonorhynchus 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).

Figure 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 absorbance 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 $0.015 \mu\text{m}^{-1}$ (Bowmaker, 1977). The spectral absorbance 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).

Figure 9: Calculated relative photon catch of the great bowerbird (*C. nuchalis*) SWS1 single cone photoreceptor combined with ocular transmission of (A) great bowerbird ($\lambda_{T0.5} 348 \text{ nm}$) and (B) a typical VS-type bird ($\lambda_{T0.5} 363 \text{ nm}$), 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° 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).

FIGURES

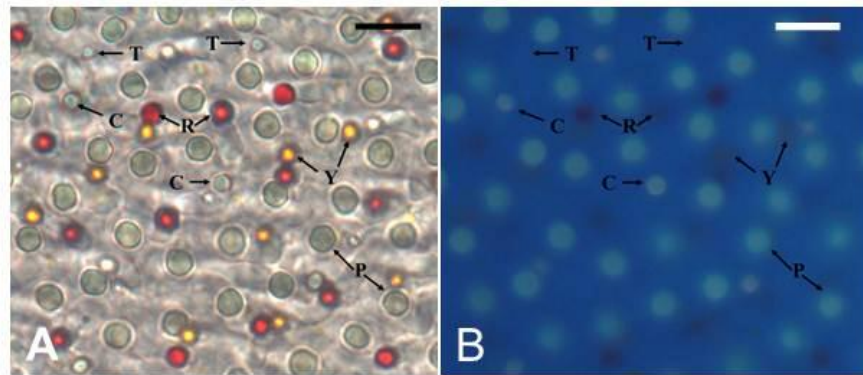


Figure 1

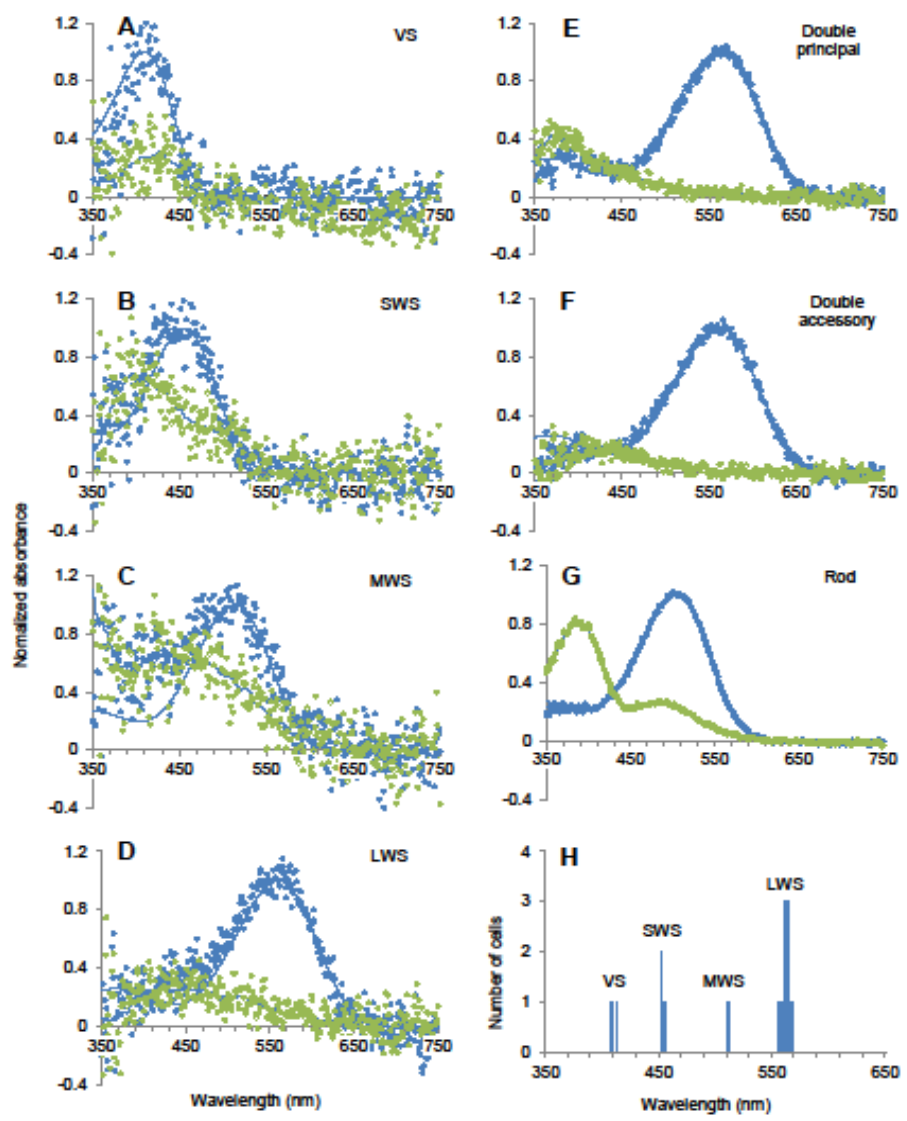


Figure 2

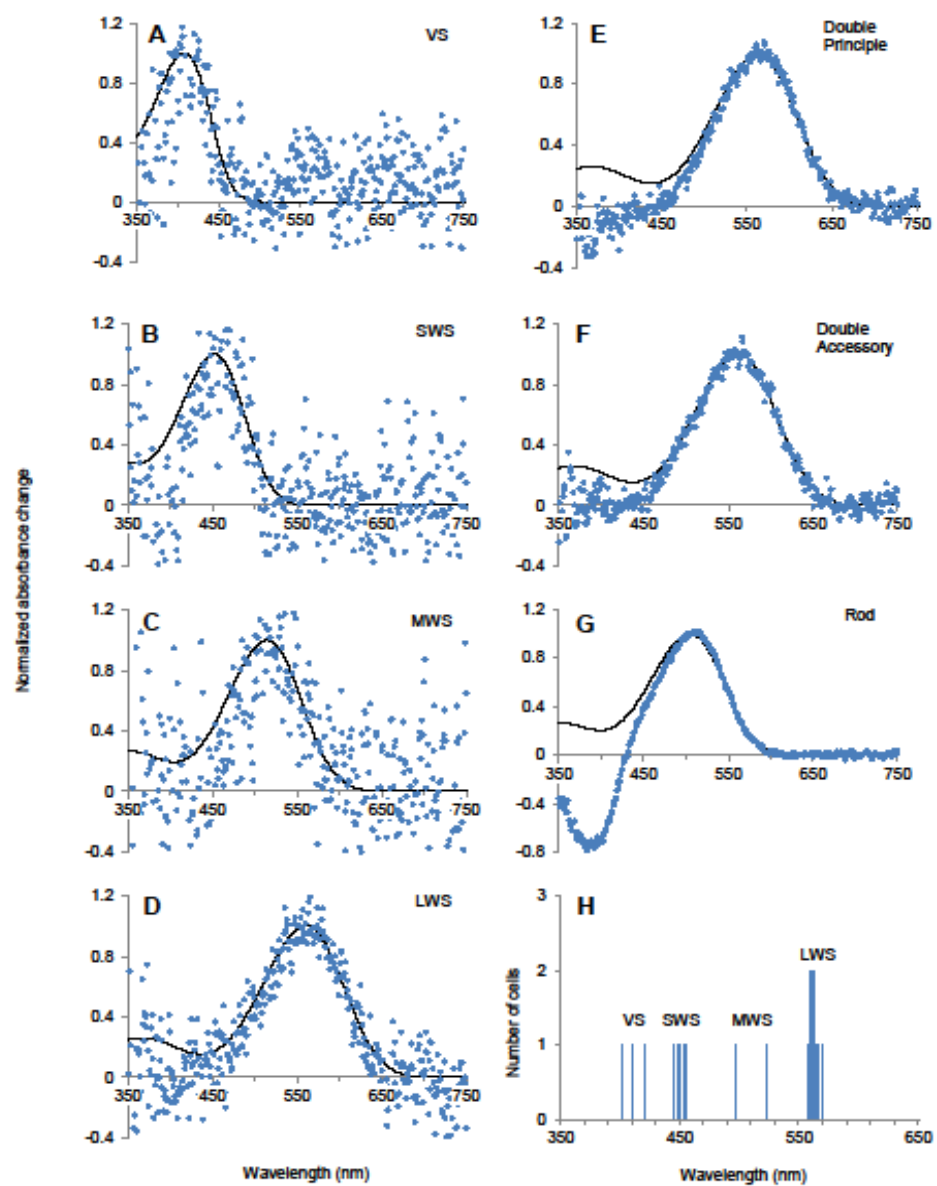
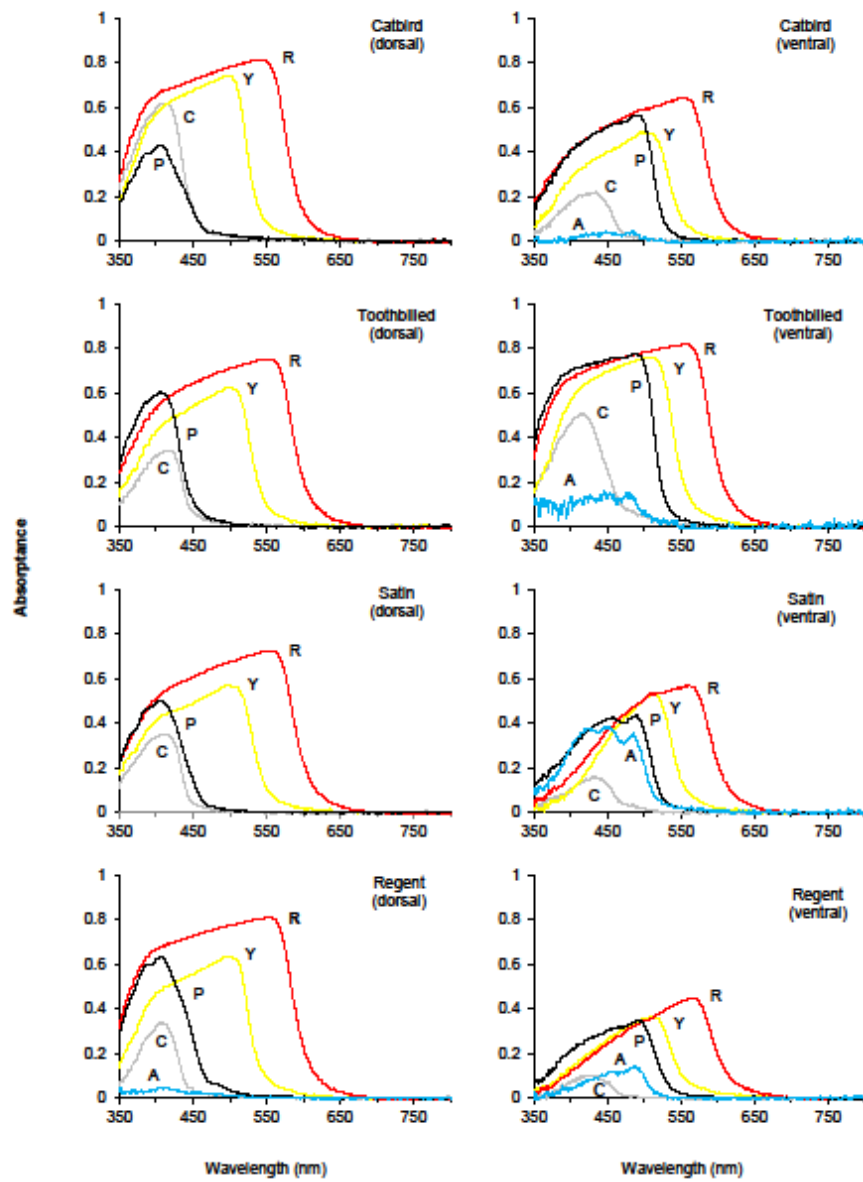


Figure 3



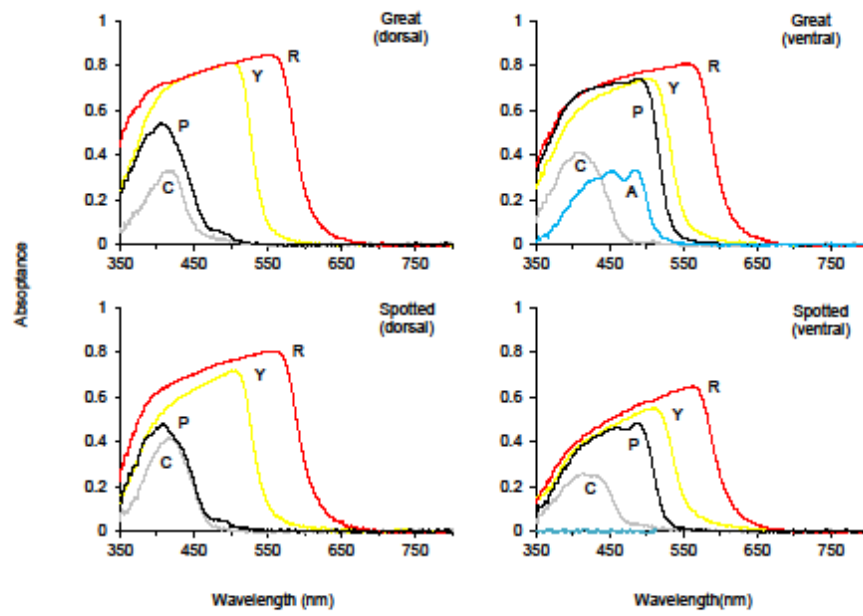
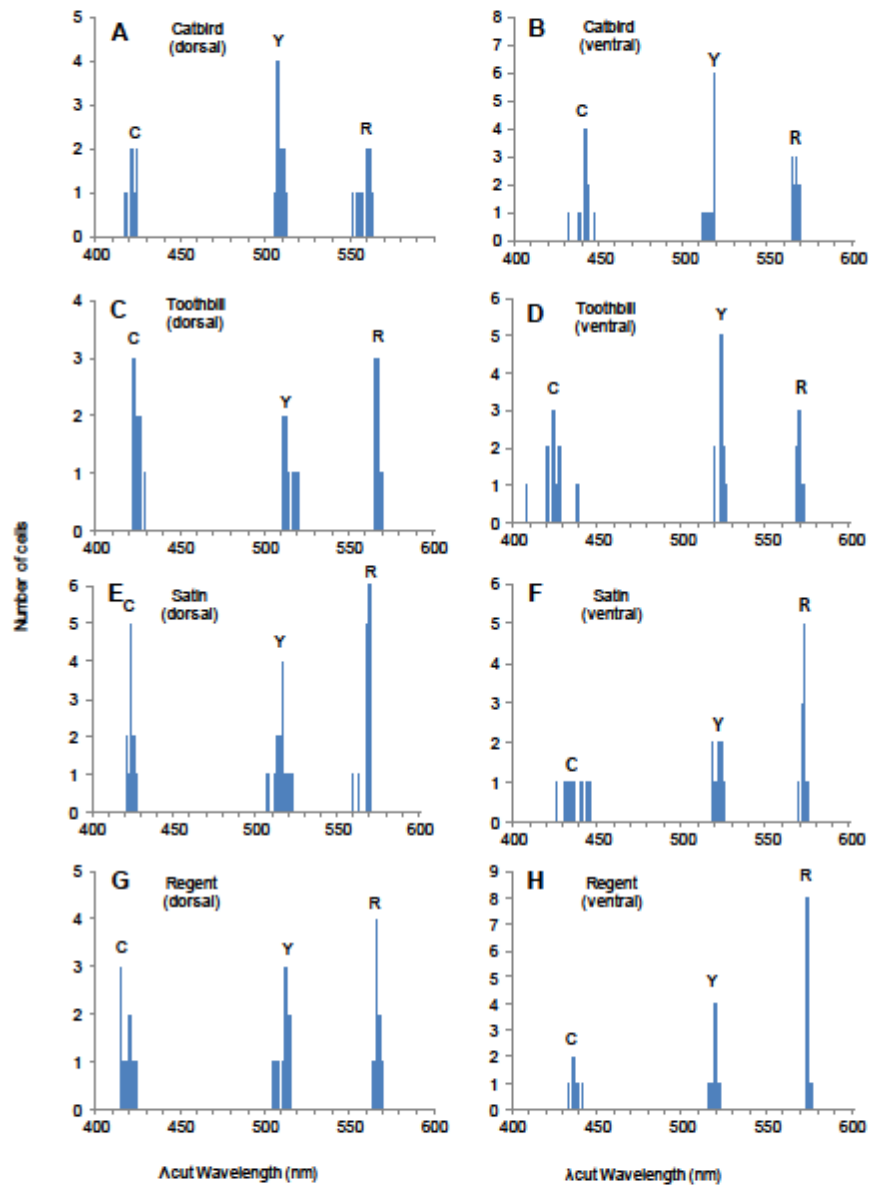


Figure 4



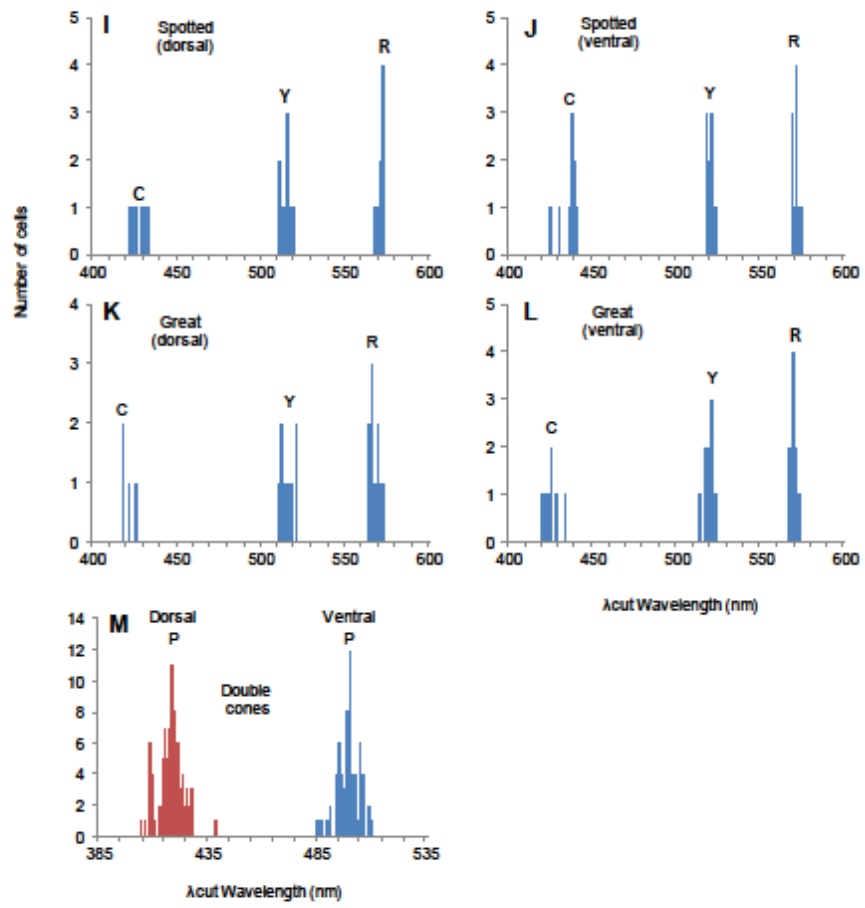


Figure 5

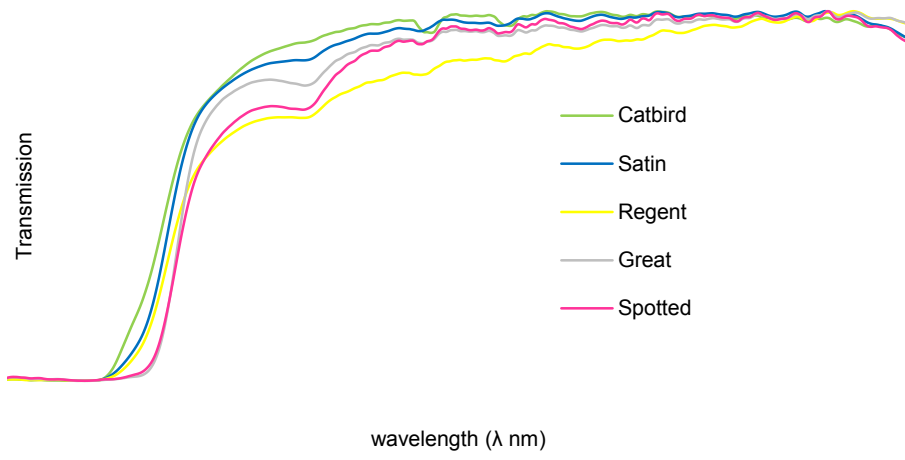


Figure 6

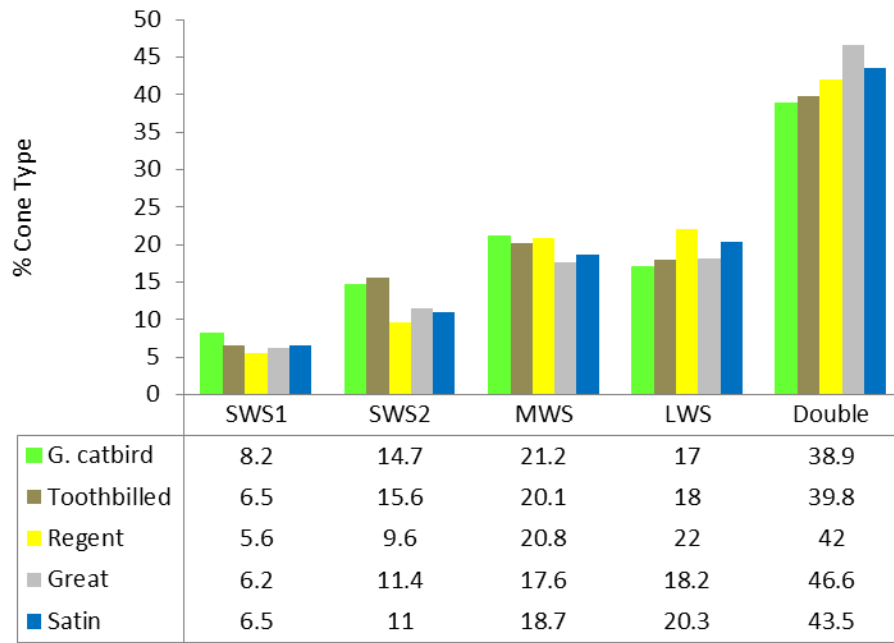


Figure 7

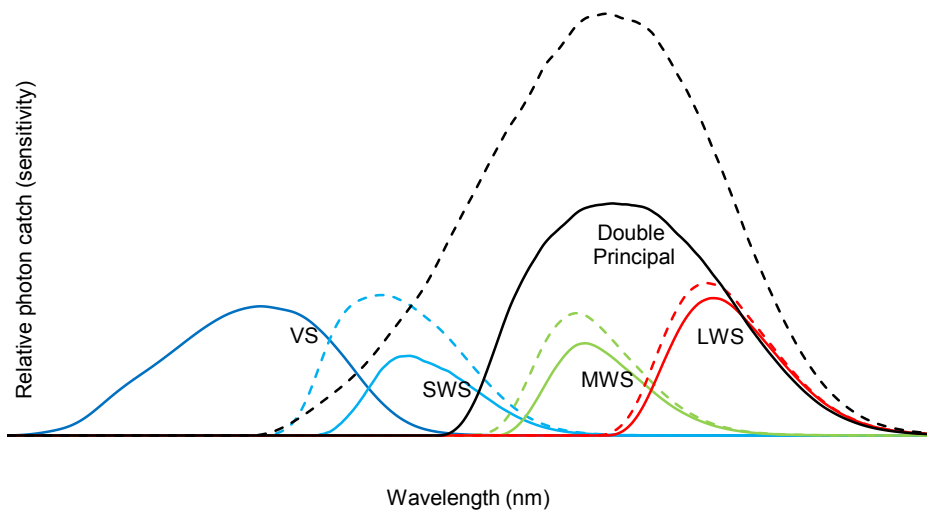


Figure 8

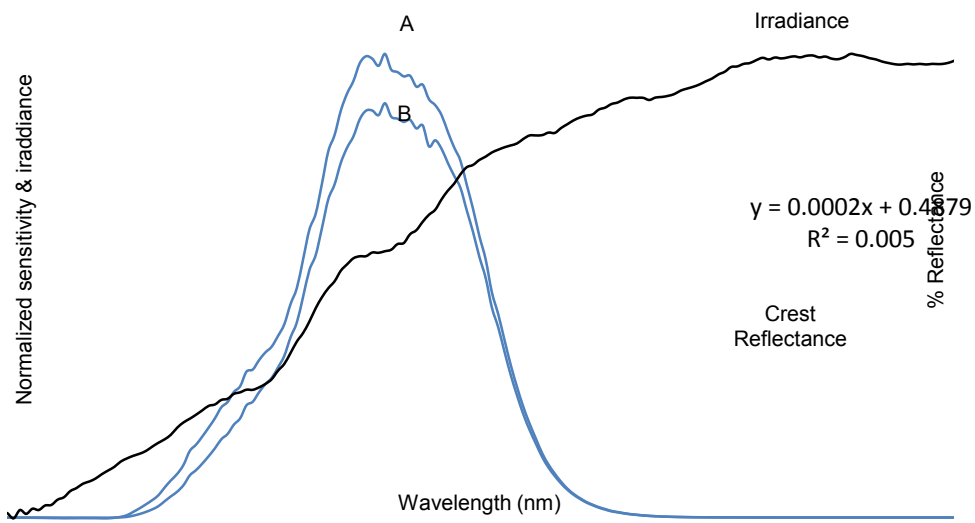


Figure 9

Chapter 2: Does learning of display traits influence frequent hybridization between two species of bowerbirds with distinctive sexual display?

ABSTRACT

Divergence of sexual displays is an important cause of reproductive isolation in birds. The degree of isolation is generally expected to scale with the amount of divergence, but there are surprising exceptions. Here we report evidence of ongoing natural hybridization between two polygynous bowerbird species, *Chlamydera maculata* and *Chlamydera nuchalis*, that are easily visually differentiated by size, plumage and many species-unique display elements. We use data on plumage, morphology, and mtDNA to discriminate hybrids from parental species birds that occupy a previously unreported contact zone in Northwest Queensland, Australia. We also examined possible causes and consequences of hybridization. In particular, we monitored courtship and display behavior to evaluate the possibility that bowerbirds of either species may learn displays from the other species, which has been found to influence hybridization in other avian hybrid systems. We report six hybrids out of 26 captured birds, bidirectional hybridization and evidence of mtDNA introgression, which indicates that some hybrids are fertile and that hybridization between these species is ongoing. Video recordings show that some pure species males in the zone of contact perform displays of the other species, suggesting that display learning between species may have occurred.

INTRODUCTION

The speciation by sexual selection hypothesis suggests that divergence of sexual displays drives behavioral reproductive isolation (Andersson, 1994; Panhuis et al., 2001; Price, 1998, Uy and Borgia, 2000). According to this hypothesis, differentiated displays render males unattractive or unrecognizable as potential mates. This may be an especially important mechanism of reproductive isolation in part because it tends to develop faster than post-zygotic incompatibility (Andersson, 1994; Coyne and Orr, 2004). In birds, behavioral isolation has special significance because development of post-zygotic isolation is relatively slow compared to many non-avian taxa (Fitzpatrick, 2004; Stanley and Harrison, 1999; Wilson et al., 1974).

Empirical and comparative studies offer support for the speciation by sexual selection hypothesis in a number of avian taxa (e.g. Panhuis et al., 2001; Morrow et al. 2003; Edwards et al., 2005). However, there are examples of hybridization between polygynous lekking species that are characterized by large display differences and extreme female choosiness, e.g. birds-of-paradise, grouse, and manakins (for review see McCarthy, 2006), which seem contrary to this hypothesis. Unfortunately, little is known about the causes of hybridization in avian taxa that experience such strong sexually selected divergence. Some commonly proposed explanations for hybridization that may apply to these taxa include that females prefer heterospecific males because they are larger or more aggressive and that females may hybridize as a last resort for reproduction when conspecific males are unavailable (Grava et al., 2012; McCarthy, 2006; Randler, 2002). Also, multiple studies have demonstrated that display learning between males of different

species can promote hybridization (e.g. Baker; Baker and Boylan, 1999; Curry, 2009; Grant and Grant, 1996; Grant and Grant, 1997a; Grant and Grant, 1997b; Qvarnstrom et al., 2006; Secondi et al., 2003; Secondi et al., 1999; Slagsvold et al., 2002).

Bowerbirds, *Ptilonorhynchidae*, are a family of polygynous, lek-like species that are highly differentiated across a suite of diverse displays providing an attractive system to examine the dynamics and causes of hybridization and reproductive isolation in the context of strong divergent sexual selection. Male displays include plumage, construction of bowers, many “dancing” and vocal courtship elements, and the collection of colored display objects, which are commonly referred to as decorations (see Borgia, 1986; Kusmierski et al., 1997). Also, there is compelling observational evidence to suggest that learning plays a prominent role in the acquisition and coordination of display elements; juvenile males frequently visit adult bowers to observe or receive display from experienced males and they congregate with other juveniles at rudimentary bowers where they reciprocate courtship display routines and “practice” bower building and decorating behavior (e.g. Borgia, 1986; Frith et al., 2004a; Marshall, 1954). Moreover, the long duration of this phase (5-7 years) and gradual display improvement over time also suggests a complex learning process.

Reports of hybridization between bowerbird species are rare, but there have been few direct investigations. To date, only four confirmed hybrid individuals have been documented among nine sympatric/parapatric pairs of species, although several hybrids have been reported between two very closely related bowerbirds, *Sericulus ardens* and *S. aureus*, that had been generally recognized as subspecies until recently (Frith et al., 2004b). Two indirect lines of evidence suggest that hybridization between many species

may be restricted by display differences. First, female bowerbirds are highly selective in mate-choice, which is demonstrated by their lengthy, multi-stage mate-choice process and large skews in male mating success (Uy et al., 2001). Because females exhibit such narrow display preference within their own population, it suggests that they would also have strong preference for con-specific males over heterospecific ones. Second, decoration choice experiments show distinct and reciprocally exclusive color preferences between species (Borgia, 1985; Borgia, 1995a; Borgia et al., 1987; Diamond, 1988; Endler and Day, 2006; Madden, 2003; Uy and Borgia, 2000). Additionally, extensive video monitoring of courtship activity (> 15 years) at dozens of satin bowerbird bowers in a location where regent bowerbirds are sympatric shows no copulation and only rare courtship between these species (G. Borgia, unpub data.). However, contrary to expectations, Frith et al. (1995) confirmed one hybrid and observed several suspected hybrids between the highly differentiated (Borgia, 1995b; Kusmierski et al., 1997) spotted bowerbird, *Chlamydera maculata*, and great bowerbird, *Chlamydera nuchalis*, near Charter's Towers Queensland, Australia. Based on these findings, they suggest that hybridization may be ongoing between these species.

Spotted and great bowerbirds are con-generic, non-sister species that are easily visually differentiated by size, plumage, and many display elements (Borgia, 1995b). Great bowerbirds have mottled gray, black, and white dorsal plumage and whitish ventral plumage and are much larger than the spotted bowerbird that has mottled brown and tan dorsal plumage and tan ventral plumage. Great bowerbird males build thick-walled bowers using only sticks while spotted bowerbirds use mostly yellow grass to build thin

walled bowers with relatively wide avenues. Borgia (1995b) also described a number of distinct behavioral courtship display elements that are unique to each species.

Confirmation of ongoing hybridization between great and spotted bowerbirds would provide an opportunity to examine factors that influence hybridization among species that have experienced strong sexually selected divergence. In this study, we used behavioral, morphological, and molecular genetic data to investigate the occurrence of natural hybridization in a contact zone between these two species, and to evaluate the possibility of inter-specific transfer of behavioral displays through learning, which could potentially influence hybridization.

METHODS and ANALYSIS

Study populations

During a field survey in Mount Isa, Queensland in 2003 we observed great and spotted bowerbirds together at bowers and common feeding sites. We also observed birds with phenotypes that were intermediate to these two species, which we suspected to be hybrids. To investigate the possibility of natural hybridization we collected data from bowerbirds within this contact zone to compare to bowerbirds of each species from allopatric locations (Table 1; see Figure 1 for species distribution map). A critical assumption of this study is that the birds sampled from allopatric locations are genetically pure and not introgressed with the other species. Thus, most of the allopatric locations from which spotted and great bowerbirds were sampled are separated by hundreds of kilometers from the closest edge of the other species' distribution, which reduces the likelihood of either genetic exchange or display learning between species.

Much of the habitat within the contact zone is characterized by dry, open scrubland where spotted bowerbirds commonly build bowers but also includes two man-made reservoirs, lakes Moondarra (circa 1958) and Julius (circa 1976), that are fringed by more densely forested habitat, which is preferred by great bowerbirds.

Research licenses and procedures were approved by Queensland Parks and Wildlife and James Cook University animal ethics committee, respectively. The Australian Quarantine and Inspection Service and USDA APHIS approved transportation of blood samples to the United States.

Blood collection, mitochondrial DNA sequencing and molecular sexing

Birds were captured near bowers and feeding sites via mist-net or cage-trap. Blood was collected via a wing venipuncture into hematocrit tubes and stored in Longmire's lysis buffer (Longmire et al. 1997). We amplified approximately 700 base pairs of cytochrome B (cytb) mitochondrial DNA (mtDNA) from sympatric and allopatric birds (Table 1). A mitochondrial loci/marker was used because it is maternally inherited and therefore useful for determining whether hybridization is unidirectional or bidirectional between species. Also, previous work showed that cytb mtDNA is approximately 6% diverged between species (Kusmierski et al. 1997; Zwiers, 2009), and no significant differences in species relationships within clades compared between cytb and NADH dehydrogenase subunit 2 topologies (Zwiers, unpub. data) makes cytb a reliable species-diagnostic marker for this study. Molecular analysis was performed at the genetics laboratory of the Smithsonian Institution National Zoological Park in Washington, DC. DNA was extracted using a Qiagen DNeasy (Valencia, CA) extraction kit. PCR was

performed using conserved primers L14841 and H15149 (Kocher et al., 1989). Sanger sequencing of amplified fragments was performed on an ABI 3100. Chromatographs were imported into sequencer version 5.0 (Gene Codes Corp., Ann Arbor, MI) to align and edit sequence data.

Sexing of birds was performed via PCR using primers P2 and P8 (Griffiths et al., 1998) to amplify the CHD gene of the Z and W chromosomes. PCR products were separated on a 2% agarose gel via electrophoresis and then analyzed visually.

Hybridity analysis

To analyze hybridity, we performed a principal components analysis (PCA) that included left wing length, right tarsus length, bill length, weight, and a composite plumage score for each bird (Figure 2; Table 1, 2 and 3) (Neff and Smith, 1979). We used descriptions and images for spotted and great bowerbirds from field guides (e.g. Simpson et al., 1999) and our own examination of captured birds to create a plumage index based on six body regions (Table 2 & Figure 2; Short, 1965). Plumage was scored on a scale of one (pure great bowerbird) through five (pure spotted bowerbird). The scores for all six regions were summed to create a composite score for each bird.

To classify birds, we plotted the first versus second principal components that together explain over 91% of total variation. Spotted bowerbirds and great bowerbirds from allopatric locations are separated by a relatively large distance on the graph, reflecting the large phenotypic differences between these species. We classified any bird from the Mount Isa contact zone that plotted in the space between the parent species clusters as a hybrid, based on their phenotypic intermediacy. Birds from the contact zone that plotted

within the phenotypic range of a parent species were classified as that species, unless they had cytb that was characteristic of the other species, which indicates mtDNA introgression.

Collection of behavioral display data

We used automated video cameras with motion detectors to record display behavior at bowers during peak mating season at all study locations (Table 4; Borgia, 1985). Males perform all display behavior at their bowers, which they typically maintain in the same location throughout the months long season. We found 14 bowers within the contact zone and recorded behavior at 10 bowers; three bowers that were located close to the shore of Lake Julius were filmed for five days and seven bowers in the southern portion of the study area were filmed for several weeks. Recordings include courtship displays to females and “practice” displays that males perform to empty bowers and with other males, including adults and juveniles (Borgia, 1986). In the field, we also hand measured bower dimensions and documented bower construction materials. However, we did not evaluate decoration object preferences to investigate display transmission because object availability, especially prized man-made objects, varied considerably across the contact zone, making it impossible to determine whether spotted and great bowerbirds in this contact zone have distinct object preferences or if they are just limited by which objects they can find.

Behavioral analysis

We analyzed video recordings of bower activity and directly observed activity at bowers to determine whether males in the contact zone (1) were visiting heterospecific bowers (where they might potentially learn heterospecific display) and, (2) if any putative parent species males were performing displays that are unique to the other species in allopatry (which might have resulted from cross-species learning). To do this, we first classified birds that were recorded in videos as either hybrid, great or spotted bowerbird. Most bower owners that were video recorded were classified based on PCA. Alternately, birds that were only recorded on video but were not captured, and thus not included in the PCA, were classified based on images of plumage and relative height. These characters are very different between species and make classification relatively easy. Height was estimated by comparing birds to reference objects of known size in the video, such as the measured height of a bower. We only included birds that we could classify phenotypically with confidence based on high quality images and close proximity to height references. After classification, we then scored the behavior of males for display elements that are unique to each parent species in allopatry following Borgia's display descriptions (1995b). Furthermore, we corroborated Borgia's (1995b) classification of species-unique display elements by analyzing all videos of spotted bowerbird courtship display that we recorded at allopatric populations in 2003 and also archival and recent videos of great bowerbirds from allopatric populations that were filmed by Borgia and Savard (unpub data).

RESULTS

Hybrid analysis

We captured 26 birds within the contact zone and classified four males and two females as hybrids based on phenotypic intermediacy (Table 5; Figure 3). Five of these hybrids had *cytb* that was characteristic of the spotted bowerbird, and one female hybrid had great bowerbird type *cytb*. Two other male birds clustered within the phenotype space of the spotted bowerbird on the PCA graph but had great bowerbird type *cytB*, providing evidence of genetic introgression. These two birds and one of the hybrids were bower owners. The hybrid bower owner had a great bowerbird type bower (i.e. thick walls made with only sticks and a relatively narrow avenue) that was located near Lake Julius, where most great bowerbirds in the contact zone were observed. The other two birds had spotted bowerbird type bowers (relatively thin grass walls and wide avenue) and were found in the southern part of the study site, where spotted bowerbirds are much more common. Of the remaining 17 captured birds, we classified 11 as spotted bowerbirds and 6 as great bowerbirds.

Behavioral analysis

Video recordings at all bowers included multiple display routines from each bower owner and displays from juveniles or non-bower owning males that visited those bowers. Video recordings from the southern portion of the contact zone, where spotted bowerbirds are most common, show spotted and great bowerbird males and hybrids visiting and displaying at spotted bowerbird bowers. The videos also show several birds of each species and hybrids attending one bower that was likely being used as a juvenile practice bower (Table 6). We observed great bowerbirds visiting a total of four spotted

bowerbird bowers. Also, two great bowerbirds were present simultaneously at two spotted bowerbird bowers and at the practice bower. However, because birds were not marked, we do not know the total number of great bowerbirds that we observed, e.g. some may have visited multiple bowers. Hybrids were observed visiting at least three spotted bowerbird bowers and the practice bower. It is possible that hybrids may have attended other bowers but the video recordings of those particular individuals were not clear enough to make a confident determination.

Also, males at multiple bowers were observed performing display routines that included unique behavioral elements from both species (Table 6). However, no adult male bower owner that was classified as pure species used more than 2 heterospecific courtship elements, comprising no more than 20% of total behavioral elements included in a display routine that was performed to another individual. In most instances, these birds only incorporated one heterospecific display element. In the northern part of the study area surrounding Lake Julius, we observed no spotted bowerbirds and only great bowerbird type display elements were used.

We did record one possible heterospecific courtship at a spotted bowerbird bower that may have resulted in copulation. The male spotted bowerbird used only con-specific display elements while courting an apparent great bowerbird female (her identity was assessed based on her appearance on video). The video was briefly interrupted at the end of courtship when copulation might have occurred, but resumed immediately after courtship to reveal the female making a distinct rapid wing flapping behavior that indicates successful copulation.

Bower phenotypes

We detected no obvious indication of heterospecific influence on bower construction. All spotted bowerbirds used yellow straw and sticks to build relatively wide avenue bowers and great bowerbirds built relatively narrow avenue bowers with sticks only. Furthermore, the hybrid and “introgressed” bower owners built bowers that matched the predominant bower phenotype in their local area (Table 7).

DISCUSSION

We report the highest frequency of hybridization ever detected between bowerbird species. We classified 6 hybrids out of 26 (23 %) bowerbirds that were captured in a contact zone between *Chlamydera nuchalis* and *C. maculata* located in Mount Isa, Queensland. We also show based on patterns of mtDNA introgression that hybridization between these species is bidirectional and historical. These findings seem to conflict with the extraordinary choosiness exhibited by bowerbird females, which begs the question of what is the importance of such complex and highly coordinated courtship systems if females hybridize with widely diverged species at relatively high frequency? We provide evidence in support of the hypothesis that male bowerbirds learn displays and may also learn from different species. These data also reveal other patterns of interaction between these two species that provide further insight into the potential causes and consequences of their hybridization.

Many species of bowerbird are excellent vocal mimics (e.g. Borgia, 1986; Frith et al., 2004b; Kelley and Healy, 2010), which reveals their vocal learning ability, but the importance of learning to the development of other displays had not been confirmed. One

way to demonstrate display learning non-experimentally in a natural population is to show that genetically “pure” males of one species express the display type of the other species. Accordingly, we observed two spotted bowerbird bower owners performing a display that is unique to great bowerbirds (both of these bower owners were classified as spotted bowerbirds based on analysis of mtDNA, plumage and morphological measurements). We also observed multiple apparent pure spotted and great bowerbird males using a combination of display elements from each species at juvenile practice bowers. These data provide some of the best direct evidence in support of the long standing hypothesis that learning is central to the acquisition and development of bowerbird displays.

Learning between species can also have important implications for hybridization. For example, in other hybrid systems it has been shown that females mate with males that have learned vocalizations of the other species (e.g. Baker; Baker and Boylan, 1999; Curry, 2009; Grant and Grant, 1996; Grant and Grant, 1997a; Grant and Grant, 1997b; Qvarnstrom et al., 2006; Secondi et al., 2003; Secondi et al., 1999; Slagsvold et al., 2002). However, less is known about how females respond to cross-species learning of non-vocal displays. More work is needed to understand the display learning process in bowerbirds and whether interspecific learning of displays may affect female mate choice.

Besides the potential significance of cross-species display learning to female mate choice our data provide insight about other possible causes of hybridization. The presence of multiple male bower owners of each species in the contact zone casts doubt on the hypothesis that female great and spotted bowerbirds hybridize only in the absence of con-specific mates; as a last resort for reproduction. Although we did not visually

confirm any unambiguous great bowerbird bowers in the southern portion of the contact zone (which would suggest they are not unavailable to great bowerbird females), we did observe multiple putative great bowerbird males visiting several bowers and at common feeding sites. We also heard great bowerbird bower advertisement vocalizations at multiple locations near Lake Moondarra which suggests that they may have established bowers close to the lake shore (time constraints did not allow us to thoroughly search for bowers in this area).

We also provide evidence of mitochondrial introgression that reveals at least some female hybrids are fertile and produce viable offspring. This evidence is based on two birds that were captured in the zone of contact and clustered within the spotted bowerbird phenotype space of the PC graph but had *cytb* sequence associated with the other species. The existence of these two individuals also suggests the potential for significant gene flow between species, which could contribute to the loss of distinct species identities, including their complex courtship display systems. It is not clear how this might impact mate choice but if females do rely on these many-signal courtship systems for honest information about male quality, as evidence suggests in other bowerbird species (e.g. Borgia, 1985; Keagy et al., 2009; Madden, 2002), then loss of reliable signaling could have significant consequences.

The extent of gene flow between species will be affected by the relative fitness of their hybrid offspring. Thus, although more extensive molecular analysis of a larger sample is required to properly evaluate the level of gene flow, it is notable that we observed a hybrid male bower owner and two introgressed males with bowers that seemed relatively fit (with respect to display ability) compared to “pure” spotted and

great bowerbirds. Each of these three birds had a well-constructed bower and generally neatly arranged decorations when subjectively compared to the bowers of pure species males in the contact zone and from allopatric locations. Also, we did not observe any obvious deficit in their behavioral displays compared to pure males of either species. Furthermore, males that possess a bower must be at least 5-7 years old and competitive enough to establish and defend a bower from other males that frequently attempt to destroy it. This is significant, because it is thought that many adult male bowerbirds never establish bowers largely due to such intense male-male competition (Borgia, 1985).

Alternately, if hybridization between great and spotted bowerbirds does result in offspring with reduced fitness (physiological deficiencies, diminished attractiveness, etc...) one potential consequence is that selection against hybrids could drive local exaggeration of species recognition characters that prevent interbreeding, i.e. reinforcement hypothesis (Dobzhansky, 1937; Coyne and Orr, 2004). Taxa that are characterized by strong selection on displays, like bowerbirds, may be predisposed to rapid character displacement upon secondary contact (Butlin, 1989; Dobzhansky, 1937; Servedio, 2004). A major prediction of the reinforcement hypothesis is that localized selection against hybridization will generate an intraspecific phenotypic gradient such that species differences are larger in areas where their breeding ranges overlap compared to areas where the two species do not co-occur. However, our data do not show this pattern. Bowerbirds captured within the Mt. Isa contact zone that we classified as pure species do not significantly differ in plumage, displays or morphology from conspecifics located in distant allopatric locations except that great bowerbird individuals captured near Lake Julius are generally larger than birds from Mount Molloy, which served as our

allopatric reference population (Figure 3). One possible explanation for this size difference is that Lake Julius great bowerbirds are derived from the larger western subspecies, *Chlamydera nuchalis nuchalis*, and not the smaller eastern subspecies, *Chlamydera nuchalis orientalis*, that occur in Mount Molloy (Table 2; Figure 3) (Frith et al. 2004). Although we cannot rule out the possibility that this size difference does reflect character displacement related to hybridization with spotted bowerbirds, if contact between these species is a relatively recent consequence of the construction of Lake Julius and Moondarra then there has been little time for character displacement of this degree to have occurred. Alternately, selection against hybridization may act to sharpen female discrimination rather than to increase differences in male appearance between species, which seem large enough already for females to easily visually distinguish a spotted from a great bowerbird.

Considering the relatively high level of hybridization and evidence for display learning between spotted and great bowerbirds, why are hybrids so rare and why is there scant evidence of display transfer between other sympatric bowerbird species? Several possible explanations for this discrepancy are that other sympatric species 1) have greater post-zygotic reproductive isolation, 2) have experienced selection against hybridization and inter-specific display learning over a much longer period of time and 3) have much more significant differences in mating systems. For example, the satin (*Ptilonorhynchus violaceus*) and toothbilled bowerbirds (*Scenopoeetes dentirostris*) are sympatric with the golden bowerbird (*Prionodura newtoniana*) but unlike spotted and great bowerbirds they are more distantly related to one another, their bower architecture differs fundamentally (avenue bower, maypole bower, and no bower, respectively (see Frith et al. 2004)) and,

correspondingly, so does their courtship display. Thus, despite obvious differences between spotted and great bowerbirds that would seem to facilitate easy species discrimination, general similarities in bower shape, display crest, and basic plumage pattern may cause females to regard males of either species as potential mates. In support of this idea, multiple hybrids have been reported between the very closely related masked (*Sericulus Aureus*) and flame bowerbird (*Sericulus ardens*) which are more similar in appearance and display to one another than spotted and great bowerbirds, however, their status as subspecies or distinct species remains controversial (Frith et al. 2004). The only other possible evidence for cross-species learning in this family is an anecdotal report of a spotted bowerbird which lived among satin bowerbirds and exhibited their unique displays, including bower construction and the primary use of blue objects as decorations (Neville, 1988).

CONCLUSIONS

We found a high frequency of hybridization between spotted and great bowerbirds in the Mount Isa contact zone. We also present evidence for possible display learning between species. More sensitive molecular analyses may uncover an even higher level of hybridization and provide important information about gene flow between these species. Further investigation of genetics and behavior in this system would provide a better understanding of modes of display transmission in bowerbirds and may reveal whether display learning between species influences hybridization, e.g. through mate misidentification. If bowerbirds are indeed transmitting displays between species through learning, then this system also offers a unique opportunity for examining complex

cultural transmission in avian taxa. Alternatively, it is possible that hybridization is the result of mate choice inexperience, e.g. mate choice copying of heterospecifics by young females, or that female mate recognition is affected by which bower or male a female first encounters after sexual maturity (Svensson et al., 2010). Additionally, our findings support an earlier suggestion that hybridization persists in a separate location; Charter's Towers, QLD (Frith et al., 1995). Considered together, these two reports of hybridization in relatively distant locations suggest that on-going hybridization between spotted and great bowerbirds is likely in any location where there is sustained contact between them. Lastly, it is worth considering the possibility that man-made lakes Moondarra and Julius may have caused or increased the frequency of hybridization between spotted and great bowerbirds in the Mount Isa area by creating or expanding a connection between species ranges. This possibility has conservation implications for understanding the effects of human impact on interactions between wild populations, particularly ones with complex mating systems.

TABLES

Table 1. Sampling locations and data types collected. We scored plumage, measured morphological traits (right tarsus length, left wing length, bill length, weight), and collected blood to sequence cytochrome B mitochondrial DNA (approximately 700 base pairs) from bowerbirds captured in the contact zone and in allopatric locations. The number of individuals for which each data type was collected are shown.

Closest city in Queensland	Latitude	Longitude	Distribution	Species present	Birds captured	mtDNA	Plumage	Morphology
Mount Isa	20°42'	139°26'	Contact zone	All types	26	24	24	24
Hughenden	20°52'	144°10'	allopatric	Spotted	9	9	9	9
Cloncurry	20°40'	140°28'	allopatric	Spotted	2	2	2	2
Boulia	22°52'	139°51'	allopatric	Spotted	2	2	2	2
Thallon	28°29'	149°29'	allopatric	Spotted	2	2	2	2
Mt Molloy	16°42'	145°20'	allopatric	Great	17	3	10	17

Table 2. Plumage scoring index. For each bird included in the plumage analysis, six body regions were scored independently on a scale from 1 to 5. A score of 1 is typical of a great bowerbird and 5 is typical of a spotted bowerbird. Scores of 2, 3 or 4 are intermediate between typical plumage characteristics of each species. Scores for all six body regions were summed to generate a final composite plumage score for each individual. Thus, plumage scores included in the analysis ranged from 6 (great bowerbird) to 30 (spotted bowerbird). These values were used in the principal components analysis.

Species	Plumage regions and descriptions						Score
	Crown	Cheek	Breast	Belly	Wing	Back	
Spotted	Streaked brown/tan	Streaked brown/tan	Mottled brown/tan	Custard	Mottled black/brown	Mottled black/brown	5
Great	All grey	All grey	All grey	Light grey	Mottled white/grey/black	Mottled white/grey/black	1

Table 3. Morphological measurements and plumage scores. Means (Standard Deviation) of phenotypic characters used in PCA for spotted and great bowerbird individuals from allopatric locations, and great bowerbirds captured in Mount Isa. The larger size of great bowerbirds in Mount Isa suggests that they are derived from the western subspecies, *Chlamydera nuchalis nuchalis*, and not the smaller eastern subspecies, *Chlamydera nuchalis orientalis*, that is represented by the allopatric sample from Mount Molloy (for discussion of intraspecific variation in phenotypes see Frith et al. 2004). Weight, wing length, bill length, and plumage are ideal species diagnostic markers because the values do not overlap between great and spotted bowerbirds. There is slight overlap in tarsus length but it is still a useful marker for differentiating between species. Morphological data presented here are consistent with all previously published measurements (see(Frith et al., 2004b). Plumage values are composite scores as described in Table 2.

Parent species	Weight (g)	L.Wing (mm)	R.Tarsus (mm)	Bill (mm)	Plumage
Spotted (13)	138 (5.8)	152 (6.4)	41.3 (6.9)	16.6 (0.58)	25-30
Mount Molloy Great (17)	190 (8.8)	168 (3.7)	46.7 (0.99)	18.8 (0.77)	6-7
Mt. Isa Great (5)	199.3 (12.7)	176.7 (7.3)	48.4 (1.5)	20.1 (1.1)	7-12

Table 4. Bowers used for analysis of bower construction, decoration display, and behavioral display (i.e. video monitoring and direct observation). Allopatric populations indicated by (A).

Location	Classification					
	Spotted bowerbird		Great bowerbird		Hybrid	
	Bowers/Dec	Video	Bowers/Dec	Video	Bowers/Dec	Video
Mt Isa	10	9	2	2	2	2
Hughenden (A)	9	9				
Bouliia (A)	3	3				
Cloncurry (A)	3					
Mt Molloy (A)			7	7		

Table 5. Factor loadings, eigenvalues, and % variance explained by each principal component.

		Principal Component				
		1	2	3	4	5
Factor Loadings	Weight	-0.959775	-0.056207	-0.130266	-0.223341	-0.093923
	Wing Length	-0.890112	0.293314	-0.305404	0.166781	-0.024093
	Tarsus	-0.856902	0.301328	0.417607	0.010667	-0.020268
	Bill Length	-0.862429	-0.473566	0.097668	0.145135	-0.036726
	Plumage	0.981251	0.058015	0.046074	0.069713	-0.163701
Eigenvalue		4.154386	0.407621	0.296299	0.103735	0.037960
% Total Variance		83.08771	8.15242	5.92597	2.07470	0.75920
Cumulative Eigenvalue		4.154386	4.562007	4.858305	4.963040	5.0
Cumulative % Variance		83.0877	91.2501	97.1661	99.2408	100

Table 6. Behavioral displays. Numbers outside parentheses indicate the total number of birds that performed either only spotted bowerbird display elements, only great bowerbird display elements or a combination of unique display elements from both species. The numbers in parentheses represent the captured birds that were classified using plumage, morphology and mtDNA. All other birds were classified visually from videos. * indicates the hybrid bower owner.

Geographic mode	Taxonomic classification	Display elements used		
		Spotted only	Great only	Spotted and Great
Sympatry	Spotted	12 (5)	0	2 (2)
	Great	0	3 (0)	1
	Hybrid	5	1 (1*)	2
	Introgressed	1 (1)	0	1 (1)
Allopatry	Spotted	24 (12)	0	0
	Great	0	7 (2)	0

Table 7. The three bowers in the contact zone that were found to be owned by the hybrid or introgressed males each resembled the locally prevalent bower phenotype in the area. Only spotted bowerbirds use grass in bower construction and their bowers tend to have wider avenues and thinner walls than great bowerbirds. Values are averages (SD) in centimeters.

Taxonomic ID of bower owner and location (# of bowers)	Bower material	Avenue entrance width	Average wall length
Great bowerbird bowers near Lake Julius (2)	Sticks only	138 (3.5)	611 (52)
Spotted bowerbird bowers near Mount Isa (8)	Mostly grass	187 (39)	542 (106)
Hybrid bowerbird bower near Lake Julius (1)	Sticks only	150	675 (21)
Introgressed bowerbird bowers near Mount Isa (2)	Mostly Grass	175 (49)	565 (106)

FIGURE LEGENDS

Figure 1. Filled circles in Panel A indicate the approximate location of bowers that were found. Panel B shows all study locations (sympatric and allopatric). In the northern portion of the contact zone near the shore of Lake Julius there are two great bowerbird bowers and a bower that belonged to a hybrid male, shown in red. Birds that were classified as spotted bowerbird were found only in the southern portion, mainly in open habitat away from the lake shore. The two red circles near Mount Isa indicate bower owners that had phenotype of the spotted bowerbird and *cytb* mtDNA sequence that was characteristic of great bowerbirds. The southern region also contained more diversity than the area near lake Julius, including one confirmed and multiple apparent great bowerbirds, at least five hybrids, and dozens of confirmed or apparent spotted bowerbirds. Furthermore, at each of two feeding sites in the southern part of the contact zone we observed several apparent great bowerbirds foraging among a larger number of spotted bowerbirds. Also, at two separate locations near the shore of Lake Moondarra we heard the unique bower advertisement vocalizations of great bowerbirds that suggest males may have built bowers in the southern region (Frith et al. 2004).

Figure 2. Images show a typical hybrid, great and spotted bowerbird and bowers of both parent species. The ruler and color card in photos were inserted by researchers for a related study. Notice the intermediate phenotype of the hybrid, particularly the color and mottling of the hybrid individual's head, breast and wing plumage.

Figure 3. Axes units are PC 1 & 2 factor loadings. Squares represent birds from allopatric locations; great bowerbirds are located on the left of graph and spotted bowerbirds on the right. Circles represent birds captured in the contact zone (6 hybrids and 2 “introgressed” birds are indicated by closed circles). Birds that plotted in the intermediate phenotype space between spotted and great bowerbirds from allopatric locations (between approximately -1.25 and 1.1 on the x-axis) were classified as hybrids. Notice that the great bowerbirds from the contact zone are larger than the ones captured in Mount Molloy. This may be due to their being derived from the larger western subspecies, *Chlamydera nuchalis nuchalis*, compared to the smaller eastern subspecies, *Chlamydera nuchalis orientalis* (for discussion of intraspecific variation in great bowerbird morphometrics see Frith et al. 2004).

FIGURES

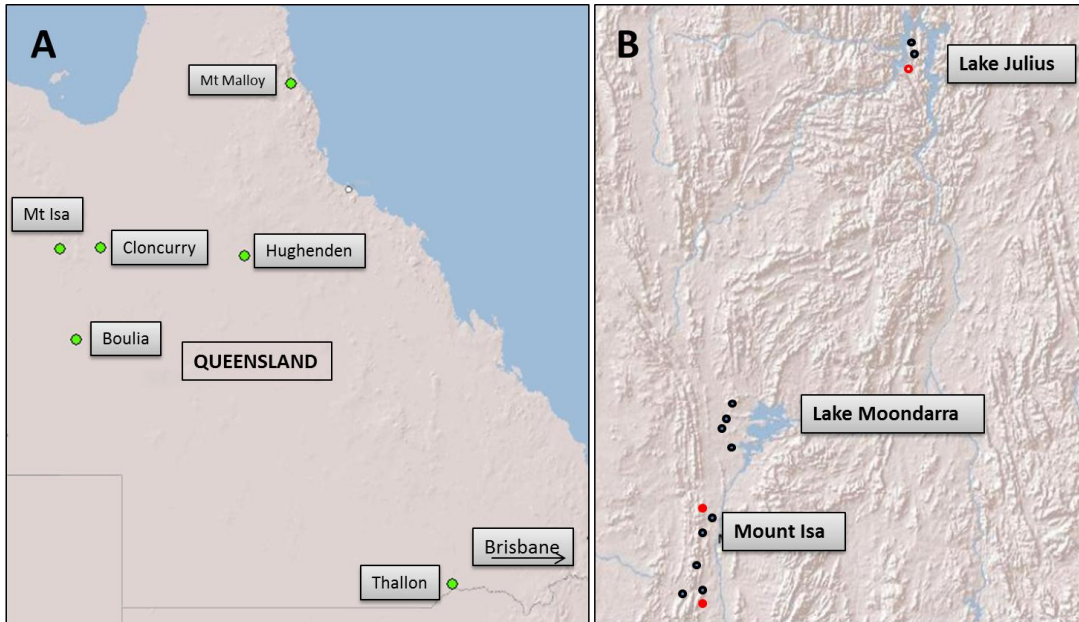


Figure 1

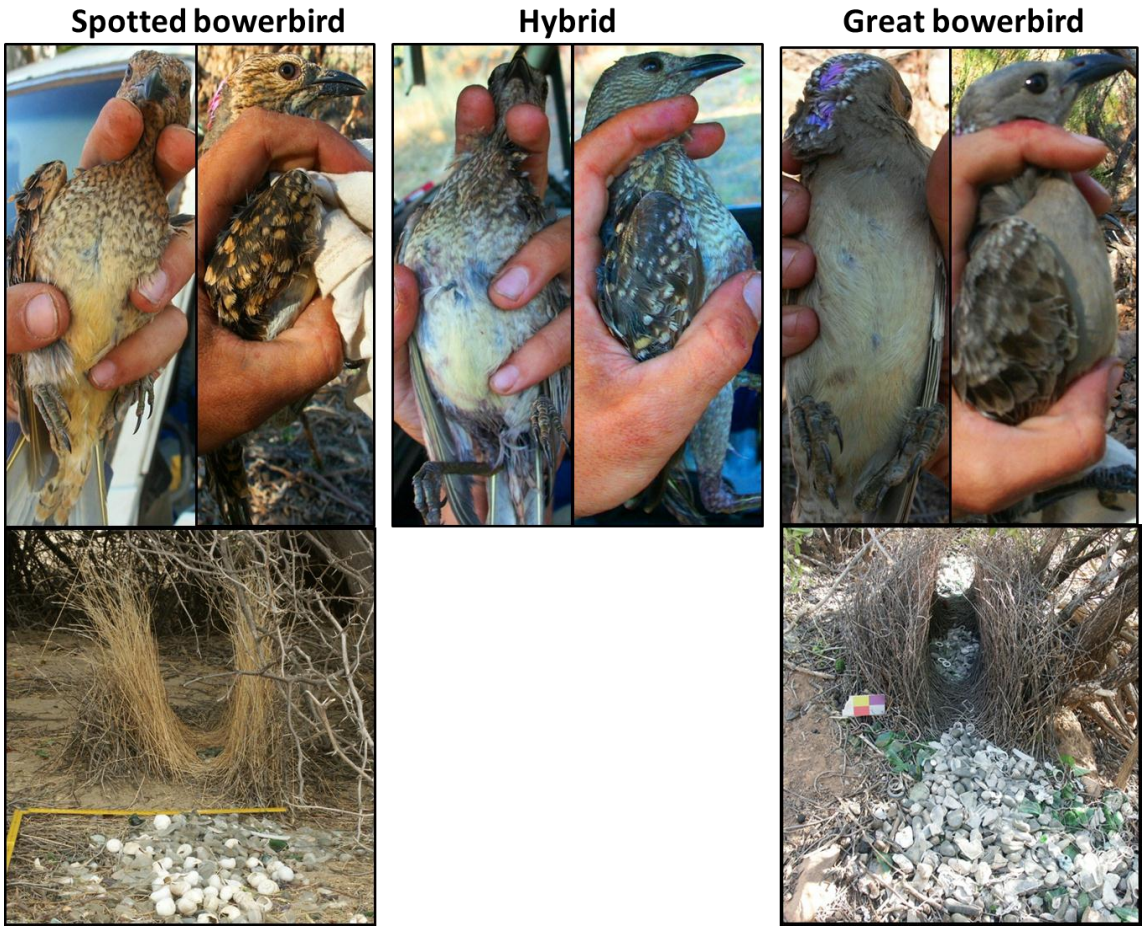


Figure 2

Chapter 3: No evidence that great, spotted or western bowerbirds create visual displays to attract females and increase mating success.

Abstract

Bowerbirds have some of the most complex sexual displays including their unique decoration displays. These displays are constructed from objects collected from the environment that males arrange at their bowers. Recent studies claim that male great bowerbirds collect certain objects to create a visual illusion called Forced Perspective that is suggested to increase male courtship success. Males supposedly create Forced Perspective by gradually increasing the size of objects with distance from the bower to make an even size gradient. However, a later study that re-examined the data found no support for this hypothesis. Here we propose an alternate hypothesis that suggests males avoid placing large obstacles close to the bower because they may interfere with their courtship display to females. To test these hypotheses, we conducted a survey of human subjects who were instructed to visually assess the size related distribution of objects in photos of bower decoration displays of great, spotted and western bowerbirds and choose among four different description of decoration pattern. We found over 50% agreement among subjects on their chosen pattern description for most bowers surveyed, indicating that this methodology is robust. There was no consistent support for any of the decoration pattern choices suggesting that the bowerbird species we tested do not create Forced Perspective or any of the other decoration patterns.

Introduction

In recent years there has been much interest among evolutionary, sensory and behavioral biologists in signal design, the form of traits used to communicate, including properties like color and pattern (see Endler 1992). Much of the research on signal design focuses on morphological or behavioral traits used in sexual display, but also includes off-body displays such as collections of objects and constructions. Some of the most extraordinary off-body displays are bower decorations collected from the habitat and displayed by males of bowerbird species, family *Ptilonorhynchidae*. Recent work has suggested that decoration displays in the great bowerbird species, *Chlamydera nuchalis*, are constructed to produce a visual illusion called forced perspective (FP) that distorts female perception and is suggested to increase male mating success (Endler et al. 2010, Kelley and Endler 2012a,b). It has been suggested that this is a cognitively sophisticated behavior that is comparable to human art. However, several authors have questioned the validity of this hypothesis (Anderson 2012, Borgia et al. 2012). Here we test the FP hypothesis and competing hypotheses to investigate the design of bower decorations.

Approximately half of the bowerbird species (10) build a bower made of two walls of vertically placed sticks or straw enclosing an “avenue” where females stand while they receive courtship from the bower owning male (Frith et al. 2004). Most decorations are placed on the display court in front of the avenue where the male performs dancing and vocal displays (Borgia 1995). Each species has a characteristic decoration pattern that strongly suggests object placement is deliberate and important (Frith et al. 2004). For example, great bowerbirds (*Chlamydera nuchalis*) have a complex decoration pattern in which males place red and green objects to the side of the bower, pieces of clear glass are

typically located close to the bower avenue entrance, and monochromatic (white and gray) objects, which comprise most of the display, are placed on the display court. Spotted (*C. maculata*) and western (*C. gutatta*) bowerbirds show similar object distribution patterns with somewhat different color usage. Additionally, studies of multiple species show that when decoration objects are experimentally rearranged, males typically respond quickly to restore their original pattern (Kelly and Endler 2012a,b).

It is likely that decoration displays have multiple functions. Borgia (1995a) suggested that decoration displays in certain species function initially from a distance to advertise the bower site to females that are flying above and searching for mates and then at close range to visually stimulate females during courtship. Also there are certain prized objects that bowerbirds frequently steal from each other and the relative number of those objects on a male's bower may reflect his dominance (Borgia et al. 1985, Borgia and Gore 1986, Madden 2002).

The recently proposed FP hypothesis suggests that great bowerbird males gradually increase the size of objects with distance from the bower to produce an even size gradient such that all objects subtend the same visual angle on the eyes of a female that is standing within the avenue (Endler et al. 2010). The supposed main effect is to make all objects appear the same size regardless of distance. Endler et al (2010) suggest that females may judge relative male cognitive quality from his ability to produce this illusion or that FP may cause other displays in the foreground to appear larger and more attractive, such as when the male displays his plumage crest at the bower entrance during courtship. To demonstrate that males are creating this illusion they measured object size by distance from the bower from pictures taken directly above the center of the display court and

analyzed the size gradient. A later study suggested that males with a more even size gradient across the decoration display have higher mating success (Kelly and Endler 2011). However, Borgia et al (2012) identified several problems with these studies. First, they claim that measuring objects from a 90° perspective may not be the appropriate way to investigate forced perspective because females that are standing within the bower avenue actually view objects from a much lower angle. Consequently, the true dimensions of objects may be obscured by other objects in the foreground. This would make the portion of the object that is visible to the female smaller than the size measured from a top down perspective. Second, Borgia et al (2012) show that Kelley and Endler's (2012a) data do not demonstrate the relationship with male mating success that they offer in support of their hypothesis, and that several of the observed trends are opposite the predicted direction. Additionally, our own observations of bower decoration displays suggest that common large or small outlier objects may interrupt the kind of even gradient that is necessary to produce the illusion.

We propose an alternate hypothesis to FP to explain the size-related pattern of object distribution on great bowerbird bowers. The obstacle avoidance (OA) hypothesis proposes that males place smaller objects in the path where males display near the front of the bower so that there are no obstacles that may interfere with movements as they perform displays. This hypothesis is based on observations of numerous videos of *Chlamydera* bowerbird courtship in which males court females while very close to the bower entrance and are able to avoid stepping on large decoration objects because they are placed farther from the bower. This hypothesis differs from what is predicted by the FP hypothesis because it does not require an even size gradient across the entire

decoration collection. OA only predicts that smaller objects will be placed very near the bower.

We asked human subjects to judge the size related distribution pattern of objects based on visual assessment of bower display courts from photos. We offered four possible responses that they could choose to describe the decoration array on great, spotted and western bowerbird bowers. The choices were that; 1) males collect objects that are all approximately the same size; 2) there is random size distribution of objects, suggesting that size distribution may not matter; 3) there is an even gradient of increasing size as predicted by the FP hypothesis, and; 4) small objects are placed close to the avenue and larger objects are farther back, consistent with the OA hypothesis. Their responses were recorded as a measure of the patterns observed. We expanded our study beyond great bowerbirds to include spotted and western bowerbirds because they have similar decorating and courtship behavior. Because males of all three species perform courtship near the bower entrance we expected that the OA hypothesis might predict patterns of decoration displays of all three species.

Methods

We surveyed 104 undergraduate students at the University of Maryland, College Park, to assess patterns of object size distribution on 18 great, 16 spotted, and 6 western bowerbird bowers from various locations across Australia. Images of great bowerbird bowers were from Darwin (12°28' S, 130°50' E), Northern Territory, Atherton Tablelands (16°68' S, 145°33' E) and Mount Isa (20°42' S, 139°46' E), Queensland. Spotted bowers were located in Hughenden (20°52' S, 144°10' E) and Thallon (28°30' S,

148°52' E), Queensland. Western bowerbird bowers were all from Alice Springs (23°41' S, 133°52' E), Northern Territory. Only a few survey participants had knowledge of bowerbirds but none had prior research experience with bowerbirds or had visited an actual bower. Subjects were provided with a multiple choice answer sheet and instructed to choose one of four descriptions that best matched the object distribution pattern of decoration displays in digital images of bowers. The images used were available from previous work and did not include size scales. Thus, the subjective methodology employed here allowed for assessment of overall size-distribution pattern where direct measurement was not possible. This approach provides a simple and straightforward way to distinguish between visual patterns that are distinctly different. Images were taken from approximately 1.25 m directly above the bower decorations display in most cases (Figure 1). In cases where access directly above the display court was obstructed by vegetation, photos were taken from an angle but these images were only included if it appeared possible to accurately judge object size distribution. In particular, FP is a conspicuous pattern that should be obvious from an angle. To avoid biasing our sample selection with regard to decoration patterns we included useable images from all bowers that we could obtain.

The multiple choice options were; A) all objects are approximately the same size; B) the size distribution of objects shows no clear pattern; objects are randomly arranged with respect to size; C) the arrangement of objects follows an *even* size gradient such that small objects are placed near the bower and the size of objects increases *gradually* with distance from the bower; D) there is a sudden, step-like change in the size of objects; small objects are placed very close to the bower and larger objects are placed away from the bower. Answer C is consistent with the FP hypothesis (Endler et al. 2010) and answer D is consistent with the OA hypothesis. To aid in assigning bower

decoration displays to the appropriate categories, subjects were provided with illustrations depicting the idealized pattern described in choices A through D (Figure 2). During the survey, each bower image was projected onto an 8' by 10' screen for 15 seconds before advancing to the next image. Subjects were instructed to focus only on the size of objects located on the display court in front of the avenue and to not consider object type, material, or color.

To assess whether decoration arrangements showed distinct patterns we used chi square tests to evaluate response consistency among subjects. We predicted that there would be a high level of agreement in response for any bower with a distinct pattern. We applied binomial distribution to test whether any of the four patterns was selected more than expected by chance across all 40 bowers and for each species. If either FP or OA exist than we predict that they should be detected on most bowers, especially given that FP should be easy to see. Research was approved by the Institutional Review Board at the University of Maryland.

Results

In assessing agreement among subjects the chi-square was significant for all bowers except two suggesting a high level of agreement in characterizing decoration distributions across all bowers (Table 1). Moreover, more than half of the subjects chose the same response for 23 of 40 bowers suggesting that the most common pattern identified for those bowers was relatively distinct. This concordance was particularly high for great bowerbirds in which more than half the subjects chose the same response for 14 of 18 bowers.

Of the forty bowers included in this study, choice A (same size), B (random), C (i.e. Forced Perspective), and D (i.e. Object Avoidance) was the most common selection for

12, 14, 4 and 10 bowers, respectively (Table 1, Figure 3). Results by species for answers A, B, C, and D are as follows; Great, 7, 5, 1, 5; Spotted, 5, 5, 3, 3; Western, 1, 4, 0, 1. We expected if either FP or OA were true that we would find a strong bias in favor of one of these hypotheses but the only pattern that was more common than expected by chance across the entire sample of 40 bowers was B, random distribution (A: $P = 0.10$; B: $P = 0.05$; C: $P = 0.98$; D: $P = 0.56$). Similarly, no pattern was chosen more than expected by chance among great bowerbird bowers (A: $P = 0.06$; B: $P = 0.28$; C: $P = 0.96$; D: $P = 0.28$) and spotted bowerbird bowers (A: $P = 0.19$; B: $P = 0.19$; C: $P = 0.60$; D: $P = 0.60$). Only B was significant for western bowerbirds (A: $P = 0.47$; B: $P = 0.005$; C: $P = 0.82$; D: $P = 0.47$) but only 6 bowers were included. Overall, FP received the least support across all bowers and among bowers of great and western bowerbirds.

Discussion

The lack of support for an FP pattern is consistent with previous work (Borgia et al. 2012) that found no evidence of size gradients or a relationship between the supposed illusion and mating success based on re-examination of data from Kelly and Endler (2012a). It is worth noting that FP was the most common response for only four bowers, including only one great bowerbird bower. Since FP geometry, as described by Endler et al. (2010), should be conspicuous, it would likely have been detectable to survey subjects if it did exist of the bowers included in this study. Although it is possible that in some areas males may be limited by the unavailability of the full range of object sizes that would be necessary to create FP, if the display is important then males would be expected to establish bowers in areas where this is not a limitation.

The FP hypothesis depends on males constructing a gradient that is observable from above and evidence that females can see the suggested illusion from within the bower. Our results do not show a gradient consistent with FP on most bowers, suggesting that the illusion does not exist for females within the bower. In a more recent study, Kelly and Endler (2012b) claim that when decoration gradients are experimentally improved, males will restore the original and less even gradient. This response, in which males intentionally diminish their own attractiveness, suggests that FP is not the goal of how males arrange decorations and conflicts with their suggestion that the FP illusion is adaptive and important for male mating success. This response also suggests that bowerbirds may not perceive the suggested illusion (Anderson 2012).

Another aspect of decoration that is inconsistent with the FP hypothesis is that bowerbirds commonly place colored decorations on top of the underlayment of monochromatic objects that is supposed to create the illusion. These decorations are often large and block the view of much of the display which would also tend to disrupt any illusion effect if it were present. It is not clear why males would create such elaborate visual illusions only to block it with other objects.

Endler et al (2010) also suggest that FP may be an example of sensory exploitation, in which males take advantage of sensory biases to manipulate females into mating. Natural selection would be expected to exert pressure to reduce or eliminate maladaptive choice through other means. One way this may happen is that females would become less influenced by or perhaps habituated to the illusion. They may avoid looking at this display and/or switch focus to other displays that are more informative and reliable. Endler et al. (2010) also suggest that males may create FP because it causes the male or

his display to look larger when appearing in the foreground. Under this scenario where the display creates a false impression of size, females may be selected to not choose mates based on size. Additionally, there is no evidence from previous studies to show that crest size does influence choice in the closely related spotted bowerbird (Madden, 2004) and Anderson (2012) argued that FP would actually make a male standing in the foreground look smaller, not larger.

We also found a lack of support for the OA hypothesis. OA suggests that males decorate with smaller objects near the bower entrance because they want to avoid obstacles that may interfere with courtship display. One reason that respondents may not have detected OA on a larger proportion of bowers may be that it requires only a relatively small area near the bower be kept clear of large objects. Thus, it is possible that subjects scored bowers as same size or randomly distributed because the vast majority of the display fit that description, whereas the collection of small objects near the avenue entrance was overlooked or not considered as a distinct feature. However, although either same size or random distribution may occur simultaneously with OA, neither would be consistent with FP.

An explanation for the selection of same size or random distribution as the most common responses for a total of 27 bowers is not readily apparent. Random distribution was the only pattern to occur more often than expected by chance, however, other studies show that males restore experimentally manipulated decoration displays back to their original pattern on bowers of male great (Kelly and Endler, 2012b) and satin bowerbirds (Larned et al. 2012), indicating that specific placement of objects is important. The response rate for random distribution was proportionally higher among Western and

spotted bowerbird bowers than great bowerbird bowers and it is possible that this portion of the decoration display may only be important in these species for attracting females from a distance (Borgia 1996) in which case a size gradient may not matter. Importantly there is also no evidence of a gradient in satin bowerbirds excepting that males place very large and bulky decorations away from the front of the bower (G. Borgia Pers. Obs). With regard to bower displays identified as same size distribution, this may reflect that preferred objects are available in a limited range of sizes or there may be a preference for objects of a particular size. Object color, size and type preference differ among bowerbird species and all species invest considerable time in decoration arrangement but further investigation is needed to better understand the function of their design.

In conclusion, we found no statistical support for FP or OA. However, because OA is consistent with either same size or random size distribution, future studies that allow consideration of these alternatives may offer support for this hypothesis.

TABLES

Table 1. Responses of survey participants to images of bower decorations. The number of participants that selected the most common response is bolded for each bower. The X^2 was not significantly different from random for only two bowers.

Species	Bower	Multiple Choice Answer				X^2_{3df}	P-value
		A	B	C	D		
Great	1	26	27	22	29	1	0.80
Great	2	1	0	88	15	202.54	>0.001
Great	3	10	82	5	7	161.31	>0.001
Great	4	44	20	31	9	26.57	>0.001
Great	5	51	17	28	8	39.77	>0.001
Great	6	73	21	5	5	119.85	>0.001
Great	7	25	59	9	11	61.69	>0.001
Great	8	47	27	22	8	30.08	>0.001
Great	9	9	5	35	55	63.54	>0.001
Great	10	22	5	24	53	45.77	>0.001
Great	11	83	13	5	3	168.77	>0.001
Great	12	28	74	2	0	136.92	>0.001
Great	13	12	1	24	67	96.39	>0.001
Great	14	55	29	11	9	52.46	>0.001
Great	15	3	2	41	58	90.54	>0.001
Great	16	98	1	5	0	266.39	>0.001
Great	17	18	74	9	3	122.54	>0.001
Great	18	12	49	29	14	33.77	>0.001
Spotted	19	18	19	56	11	47.62	>0.001
Spotted	20	24	44	21	15	18.23	>0.001
Spotted	21	75	18	6	5	127.15	>0.001
Spotted	22	7	86	6	5	184.69	>0.001
Spotted	23	87	12	5	0	193.61	>0.001
Spotted	24	21	31	29	23	2.61	>0.001
Spotted	25	28	22	39	15	11.92	>0.001
Spotted	26	34	36	23	11	15.31	>0.001
Spotted	27	13	8	30	53	47.62	>0.001
Spotted	28	38	0	35	31	35.62	>0.001
Spotted	29	96	5	3	0	248.81	>0.001
Spotted	30	7	12	10	75	123.62	>0.001
Spotted	31	35	7	12	50	46.69	>0.001
Spotted	32	84	9	4	7	173	>0.001
Spotted	33	15	80	8	1	153.31	>0.001
Spotted	34	15	34	49	6	42.85	>0.001
Western	35	19	28	25	32	3.46	0.33
Western	36	45	48	10	1	66.38	>0.001

Western	37	3	52	39	10	62.69	>0.001
Western	38	20	81	3	0	164	>0.001
Western	39	9	88	7	0	198.85	>0.001
Western	40	54	4	17	29	52.23	>0.001
TOTAL		12	14	4	10	5.6	0.13

FIGURE LEGENDS

Figure 1. Examples of bower decoration displays of spotted bowerbird (A) and great bowerbird (B).

Figure 2. Illustrations of object distribution patterns provided to survey participants.



Bower decoration displays of spotted bowerbird (A) and great bowerbird (B).

FIGURE 1

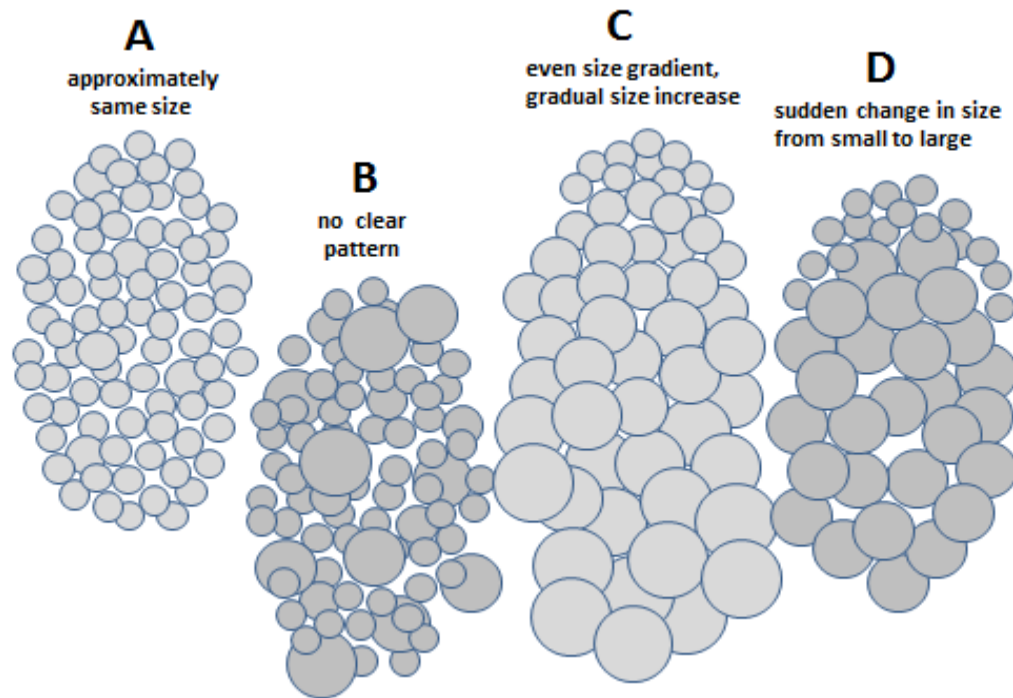


Figure 2

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