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RESEARCH ARTICLE

How parrots see their colours: novelty in the visual pigments of *Platycercus elegans*

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SUMMARY

Intraspecific differences in retinal physiology have been demonstrated in several vertebrate taxa and are often subject to adaptive evolution. Nonetheless, such differences are currently unknown in birds, despite variations in habitat, behaviour and visual stimuli that might influence spectral sensitivity. The parrot *Platycercus elegans* is a species complex with extreme plumage colour differences between (and sometimes within) subspecies, making it an ideal candidate for intraspecific differences in spectral sensitivity. Here, the visual pigments of *P. elegans* were fully characterised through molecular sequencing of five visual opsin genes and measurement of their absorbance spectra using microspectrophotometry. Three of the genes, *LWS*, *SW1* and *SWS2*, encode for proteins similar to those found in other birds; however, both the RH1 and RH2 pigments had polypeptides with carboxyl termini of different lengths and unusual properties that are unknown previously for any vertebrate visual pigment. Specifically, multiple RH2 transcripts and protein variants (short, medium and long) were identified for the first time that are generated by alternative splicing of downstream coding and non-coding exons. Our work provides the first complete characterisation of the visual pigments of a parrot, perhaps the most colourful order of birds, and moreover suggests more variability in avian eyes than hitherto considered.

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INTRODUCTION

Systematic differences in visual photobiology within species have been observed in many taxa, including teleosts (Parry et al., 2005), New World monkeys (Mollon et al., 1984) and humans (Nathans et al., 1986). This phenomenon, however, has yet to be demonstrated in birds. Currently, colour vision and visual processes are considered to be highly conserved among avian taxa (Bennett and Thery, 2007; Hart and Hunt, 2007). This general conjecture, however, is at odds with the wide diversity of niches, habitats, visual stimuli, behaviours and light environments of birds, all of which are factors that theory suggests should influence spectral sensitivity in animals (Lythgoe, 1979; Endler, 1992), and which have been shown to do so in non-avian taxa. Accordingly, it is necessary to identify candidate bird species in which intraspecific differences in visual photobiology are likely to be present.

One promising species is *Platycercus elegans* Gmelin 1788 (Aves; Psittaciformes). This parrot shows extreme variation in plumage coloration between subspecies, ranging from deep crimson (in *P. e. elegans*) to pale yellow (in *P. e. flaveoulus*), with no reported sex differences in this character in any subspecies (Forshaw and Cooper, 2002). *Platycercus elegans elegans* occupies mesic wooded and forest habitats, and *P. e. flaveoulus* riparian habitats (Forshaw and Cooper, 2002), and the distribution of the two overlaps in a hybrid zone (Joseph et al., 2008). The complex also includes the phenotypically intermediate and clinally varying *P. e. adelaidae* subspecies, in which there is much plumage colour variation between individuals in the same local area (Forshaw and Cooper, 2002; Joseph et al., 2008; Berg and Bennett, 2010). *Platycercus elegans* is perhaps the most colour variable of the ~350 species of parrot worldwide, and Mayr (Mayr, 1963) considered the species an example of a circular overlapping or 'ring' species (Irwin and Irwin, 2002), of which there are few worldwide. Recent studies of the species' population genetics (Joseph et al., 2008) and vocalisations (Ribot et al., 2009; Ribot et al., 2011; Ribot et al., 2012; Ribot et al., 2013), however, have revealed a more complex phylogeography than proposed in an 'ideal' ring species, including findings of cryptic genetic change coinciding with abrupt clines in learned acoustic parameters (Ribot et al., 2012). Of particular interest are the selective pressures driving and maintaining the evolution of plumage colour variation, many of which remain elusive (Berg and Bennett, 2010). Nevertheless, sensory drive theories (Endler and McLellan, 1988; Bennett et al., 1994), in combination with predictions arising from the known differences in habitats and their light environment and background coloration (Lythgoe, 1979; Endler, 1992), would suggest that it is plausible that intraspecific differences in vision may have evolved in the species complex. In particular, we hypothesize that visual differences exist (1) between subspecies that vary in light environment and background coloration

of their habitats and/or (2) between individuals within the highly variable subspecies *P. e. adelaidae*.

To date, knowledge of parrot vision has been mainly based on studies of one species, the budgerigar *Melopsittacus undulatus* (Berg and Bennett, 2010; Knott et al., 2012). However, these studies are incomplete, with only three out of a possible five visual pigment (opsin) classes sequenced, namely rod opsin (*RH1*) and two cone opsins [cone rhodopsin-like 2 (*RH2*) (Bowmaker et al., 1997) and short-wavelength-sensitive 1 (*SWS1*) (Wilkie et al., 1997)]. Extensive behavioural experiments using colour mixing support the physiological data derived from microspectrophotometry (MSP) (Goldsmith and Butler, 2005). In addition, *M. undulatus* has been used to establish the opponent mechanisms between cones that are used in avian colour perception (Goldsmith and Butler, 2005). The budgerigar has also been used extensively in studies of mate choice and fluorescence (Pearn et al., 2003a; Pearn et al., 2003b). In other parrots, including *P. elegans* (Carvalho et al., 2011), studies have been largely restricted to the prevalence of UV sensitivity in the Psittaciformes, which currently appears to be ubiquitous (Wilkie et al., 1997). Given that Psittaciformes are arguably the most colourful order of birds, further investigation of parrot colour vision is warranted to ensure a more complete understanding of avian visual ecology and the evolutionary mechanisms that shape it.

Here, we assessed the molecular and physiological basis of the opsins and visual pigments that serve colour vision in *P. e. adelaidae*, the most colour-variable subspecies of *P. elegans*. Firstly, we used MSP to gather *in situ* measurements of visual pigment absorbance spectra for all five pigment classes. Secondly, we obtained DNA sequences of the opsins that are expressed in the rods and cones of this species. Opsins are G protein-coupled receptors (GPCRs) that are rendered light sensitive by the Schiff base linkage of the chromophore, retinal, to a lysine residue at site 296 (Yokoyama, 2000a; Davies et al., 2012). In the opsin, amino acids present at specific sites are known to 'tune' the peak spectral sensitivity of the visual pigment to particular wavelengths (Yokoyama, 2000a; Davies et al., 2012); variation at these tuning sites may underlie, therefore, any intraspecific variation in absorbance spectra in *P. elegans*. The *SWS1* opsin gene sequence of *P. elegans* has been reported previously and has been shown to encode a UV-sensitive pigment (Carvalho et al., 2011). The types of visual pigments expressed in rods and in the other three cone classes of *P. elegans* are unknown and form the basis of this study, which is the first to identify and characterise the genetic basis of colour vision in a parrot species. Such data from multiple individuals can then be combined with cone oil droplet spectral information (Knott et al., 2010) and findings on the influence of dietary carotenoid on visual abilities in *P. elegans* (Knott et al., 2010) to determine the degree of phenotypic plasticity in vision within this species, and how colour perception and colour spaces might differ between individuals.

MATERIALS AND METHODS

MSP of four visual pigments

The spectral sensitivities of retinal photoreceptors were assessed in six *P. elegans* individuals (subspecies *P. e. adelaidae*) using microspectrophotometers at both the UCL Institute of Ophthalmology, London, UK (four captive-bred birds), and Deakin University, Geelong, Australia (two wild-caught birds). Subjects were dark adapted for at least 1 h before euthanasia. Retinal tissue samples were prepared for MSP under dim red light or infrared light using methods reported previously (Knott et al., 2010). Spectral analysis was undertaken on two computer-controlled MSP systems (Liebman and Entine, 1964; Bowmaker et al., 1997): (1) a modified Liebman dual beam (UCL Institute of Ophthalmology) and (2) a single-beam device (Deakin University). Measuring beams were aligned to pass transversely through the photoreceptor outer segment and run in 2 nm intervals from 750 to 350 nm, then back from 351 to 749 nm. After each measurement, the pigment was bleached with white light and the outer segment was rescanned to confirm the post-bleaching disappearance of the pigment and appearance of short-wavelength-absorbing photoproducts. The peak absorbance spectra (λ_{max}) were calculated from the averaged spectrum using a standardised computer program as described previously (Bowmaker et al., 1997).

In vitro calculation of the λ_{max} for the SWS1 visual pigment

The dark and acid-treated raw spectral data were determined previously (Carvalho et al., 2011) and reanalysed according to methods recently outlined (Davies et al., 2011). In brief, the previous result was analysed using the data obtained from the right-hand limb of the absorbance difference spectrum only. Often the spectral peak determined in this way yields a value that is short-wavelength shifted and does not optimally fit the rhodopsin template (i.e. with a less than optimal R^2 value). If this is the case, a better fit may be achieved if data are analysed that derive from the right-hand limb, the peak and part of the left-hand limb to a bespoke value where the R^2 value for the best fit is at its maximum.

PCR amplification and sequencing of opsin coding regions

The eyes from four (three adults and one juvenile) *P. e. adelaidae* were enucleated immediately following death, pierced, and placed in RNA*later* (Ambion, Mulgrave, VIC, Australia) for 24 h at 4°C, then stored at 20°C before shipping to the UK, where they were stored at 4°C. Muscle tissue was excised within 1 h of death and stored at 4°C in 70% ethanol, prior to genomic DNA extraction as described previously (Davies et al., 2009a).

Retinal mRNA was extracted using the QuikPrep Micro mRNA Purification Kit (GE Healthcare Life Sciences, Little Chalfont, UK) and used to synthesise cDNA using the 5′/3′-Rapid Amplification of cDNA Ends (RACE) (2nd Generation) Kit (Roche Diagnostics, Burgess Hill, UK). Partial fragments of LWS, SWS2, RH2 and RH1 coding sequences were PCR amplified using degenerate primers (AOAS and DIAP sets) and a nesting/hemi-nesting PCR protocol outlined previously (Davies et al., 2009b), prior to sequencing by standard Sanger sequencing methods. From these partial sequences, specific 5′-end and 3′-end primers (supplementary material Table S1) were designed to extend the coding sequences for the *P. elegans* opsin genes (GenBank accession nos. KF134487–KF134493) using the RACE kit detailed above.

Phylogenetic analysis

Extended or full-length sequences that encode the long-wavelength-sensitive (LWS), SWS1, short-wavelength-sensitive 2 (SWS2), RH2 (medium isoform) and RH1 visual pigments of *P. elegans* were compared with visual opsins derived from other vertebrates, specifically those determined in reptiles, birds and mammals, to produce codon-matched nucleotide sequence alignments by ClustalW (Higgins et al., 1996), and were manually refined. The nucleotide sequence for chicken (*Gallus gallus*) vertebrate ancient (VA) opsin (GenBank accession no. GQ280390) was used as an outgroup. Estimation of evolutionary distances between nucleotide sequences were determined using the MEGA Version 4.0.2 computer package (Tamura et al., 2007) and applying the maximum composite likelihood (MCL) method (Tamura and Nei, 1993), with pairwise deletion and a homogenous pattern of substitution.

Subsequently, a neighbour-joining (NJ) phylogenetic tree (Saitou and Nei, 1987) was generated with bootstrapping of 1000 replicates.

Genome mining and bioinformatics prediction of putative protein motifs

To identify *RH2* exon 5b in other bird species, a *P. elegans* genomic fragment containing exons 4a–5b was used for BLAT and BLASTN searches of the Ensembl genome databases (http://www.ensembl.org/index.html) of chicken (*Gallus gallus*, Galgal4 assembly) and turkey (*Meleagris gallopavo*, UMD2 assembly). Online bioinformatics programs were used to identify residues within the carboxyl termini of RH1 and RH2 visual pigments that are predicted to be critical for pigment function. These included: (1) CSS-Palm Server 3.0 (http://csspalm.biocuckoo.org/online.php), to determine any putative palmitoylation sites for the tethering of visual pigments to the plasma membrane; and (2) NetPhos Server 2.0 (http://www.cbs.dtu.dk/services/NetPhos/), to predict the presence of phosphorylation sites involving serine (S) and threonine (T) residues that regulate pigment deactivation by photoreceptor-specific kinases. In addition, the extended carboxyl terminus of *P. elegans* RH1 was subjected to PROSITE analysis, which searched for known protein motifs (http://myhits.isb-sib.ch/cgi-bin/motif_scan).

Ethics statement

Animal studies in the UK were carried out under the Animals (Scientific Procedures) Act 1986 in accordance with protocols approved by the Animal Ethics Committee of the University of Bristol. Retinal RNA was collected under licence from wild animals caught near Oakbank, Mt Lofty Ranges, South Australia (34°59′S, 138°50′E). Animal work in South Australia was performed under permit from the Department of Environment and Natural Resources, South Australia. Animal work in Victoria was performed with approval from the Animal Ethics Committee of Deakin University, and under permit from the Department of Sustainability and Environment, Victoria.

RESULTS

Spectral sensitivities of *P. elegans* visual pigments

The classes of photoreceptors within the retinae of birds are highly conserved, with all species studied to date possessing rod photoreceptors, double cones comprising principal and accessory components, and four spectral classes of single cones that contain oil droplets (Hart and Hunt, 2007). Birds have generally retained all four classes of cone visual pigments and thereby the potential for tetrachromacy: the LWS pigment is expressed in both members of the double cones as well as in single cones, and the other three cone pigment classes, RH2, SWS2 and SWS1, are each expressed in single cones (Hart and Hunt, 2007). Using MSP, the absorbance spectra of three cone types and a single rod class were shown to have λ_{max} values at 440 nm (short-wavelength sensitive, SWS), 509 nm (middle-wavelength sensitive, MWS) and 567 nm (LWS) and 510 nm, respectively (Fig. 1A–D). Recordings from multiple individual cells were made for MWS and LWS cones, as well for the rods. The SWS cone classes are notoriously difficult to find and the retina of *P. elegans* was no exception, with just a single SWS2 and no UV-sensitive cones found. Nonetheless, *P. elegans* had been shown previously to express an *SWS1* gene encoding a UV-sensitive pigment with a λ_{max} value at 363 nm (Carvalho et al., 2011). When reanalysed using a modified approach (Davies et al., 2011), where the data from the right-hand limb to 19 nm below the peak of the left-hand limb were considered, the λ_{max} value of the SWS1 pigment expressed *in vitro* was calculated to be 365 nm, accompanied by an increase in the R^2 value for the best fit to the template from 0.9820 to 0.9982 (Fig. 1E), a result that is consistent with previous studies of avian UV-sensitive cones (Hunt et al., 2007; Carvalho et al., 2011). Therefore, we conclude that *P. elegans* expresses five visual pigments in its retinal photoreceptors that provide coverage across the entire visual spectrum (Fig. 1F).

Identification of the visual opsin classes expressed in the *P. elegans* retina

The peak spectral sensitivities of photoreceptors vary between species such that it is not always clear which opsin gene class is responsible [e.g. MWS cones may contain either an SWS2, an RH2 or an LWS pigment (Davies et al., 2012)]. Therefore, it is important to establish the genetic basis for the different cone classes present in the *P. elegans* retina. Initially, partial coding sequences for all five visual opsins (LWS, SWS1, SWS2, RH2 and RH1) were obtained by nested/hemi-nested PCR amplification of retinal-derived cDNA using two major sets of degenerate primers. Where appropriate, these sequences were subsequently extended by 5′- and 3′-RACE to yield coding sequences of sufficient length to determine both the type of opsin expressed and the residues at key tuning sites. In all cases, the partial or full-length sequences obtained comprised five (for *SWS1*, *SWS2*, *RH2* and *RH1* opsin genes) or six (for the *LWS* opsin gene) exons [GenBank accession nos: KF134493 (LWS); KF134492 (SWS1); KF134491 (SWS2); KF134488–KF134490 (RH2); and KF134487 (RH1)].

The identity of the five *P. elegans* coding regions was confirmed by phylogenetic analysis after alignment with the visual opsin nucleotide sequences of other vertebrates (supplementary material Fig. S1).

Alternative splicing of visual opsins

The presence of alternatively spliced variants giving rise to opsin proteins with differing carboxyl termini is relatively commonplace for non-visual opsins such as VA and melanopsin (OPN4) (Davies et al., 2010). However, alternative splicing has not been reported for visual opsin genes, with variation amongst transcripts being limited to the differential use of polyadenylation signals in both human and murine *RH1* opsin genes to yield different 3′-untranslated regions (3′-UTRs) (al-Ubaidi et al., 1990; Applebury et al., 2000; Wistow et al., 2002). With one exception, only single transcripts were found using 3′-RACE in the *P. elegans* retina for *LWS*, *SWS1* (Carvalho et al., 2011) and *RH1* genes. Unfortunately, the paucity of *SWS2*-expressing cones and the resultant low levels of SWS2 mRNA transcripts meant that 3′-RACE was not successful despite multiple attempts. The exception was the RH2 transcript, for which three distinct variants were identified that arose from alternative splicing of protein-coding exons and intron retention (Fig. 2A–D), as found in many non-visual opsins (Davies et al., 2010). Specifically, ‘normal’ RH2 transcripts spliced from traditional exon 4 (named exon 4a) to traditional exon 5 (designated exon 5a) were found that encoded an opsin protein pigment (‘RH2-medium’ or RH2M) that is almost identical to that of other avian RH2 opsins. A second transcript retained intron 4 (201 nucleotides) between exons 4a and 5a, which produced a shorter protein because of a predicted stop codon located in the proximal end of the intron (‘RH2-short’ or RH2S); the extension of exon 4a into intron 4 is labelled 4b. The final transcript was unusual in that it spliced from exon 4a to a fifth exon (designated exon 5b) located 740 nucleotides downstream of exon 5a, resulting in an extended polypeptide sequence (‘RH2-long’ or RH2L) (Fig. 2A–C). Interestingly, when

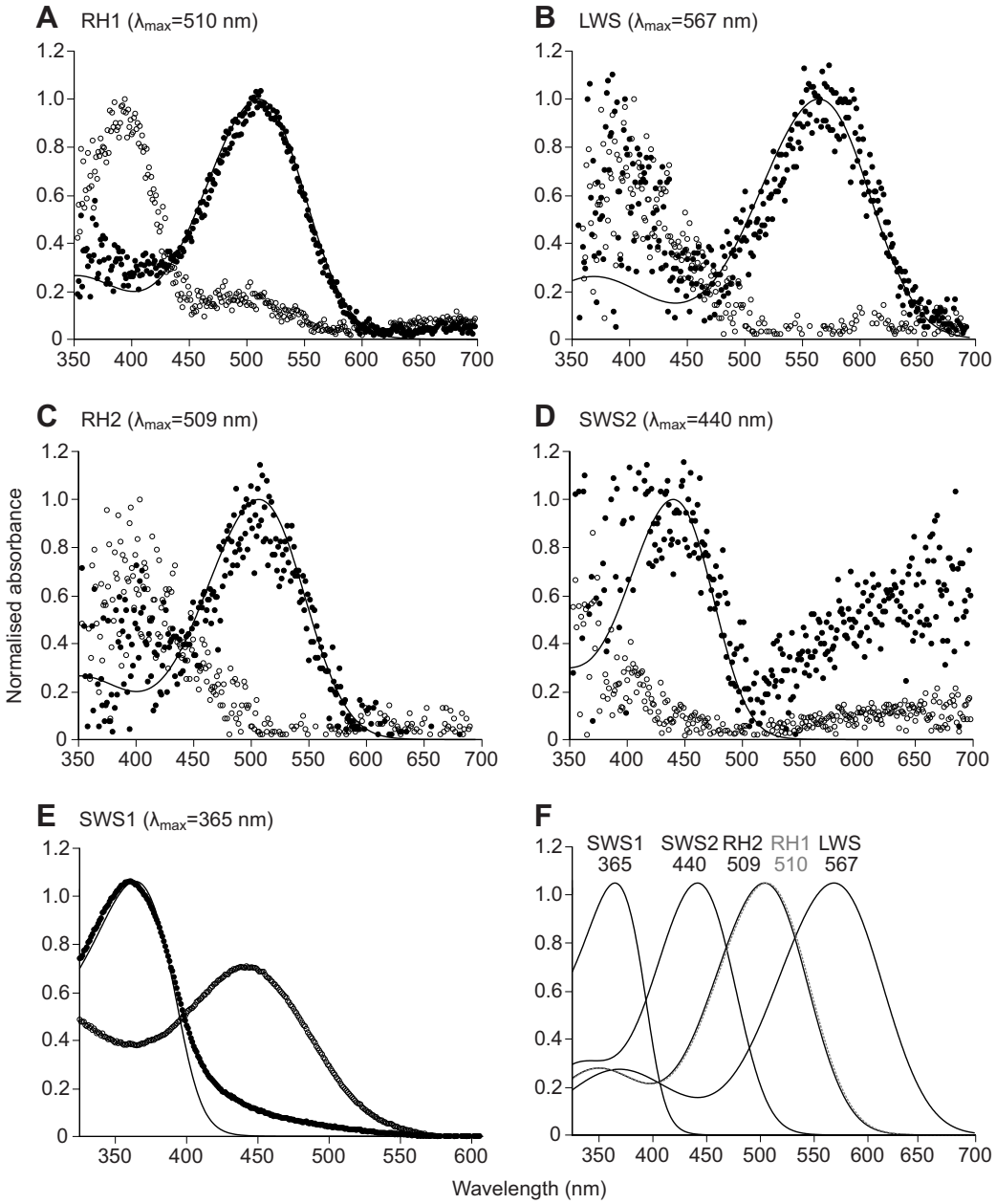

Fig. 1. Microspectrophotometry (MSP)-determined mean absorbance spectra of (A) RH1, (B) LWS, (C) RH2 and (D) SWS2 visual pigments of *Platycercus elegans* before (open circle) and after (closed circle) photobleaching by exposure to broad-spectrum white light. (E) Absorption spectra of the regenerated *P. elegans* SWS1 visual pigment. These data were obtained previously (Carvalho et al., 2011) and are reanalysed here, showing dark (closed circle) and acid-denatured (open circle) spectra. All MSP and *in vitro* regenerated dark spectra were fitted with a standard Govardovskii rhodopsin template (black line) (Govardovskii et al., 2000) to determine the spectral peak of absorbance (λ_{max}). (F) Summary diagram showing the range of visual pigments and their spectral sensitivities expressed in the retinae of *P. elegans* (cone pigments, black line; rod pigment, grey dotted line).

compared with the genomes of other birds, exon 5b, which is 13 amino acids longer in *P. elegans*, was shown to be conserved in both chicken (*Gallus gallus*) and turkey (*Meleagris gallopavo*) genomes, including upstream pyrimidine tract [$(Py)_n$] and acceptor splice site (GAG) motifs and a putative downstream polyadenylation termination signal (AAUAAA), all of which suggest that transcripts containing exon 5b may be commonly expressed in birds (Fig. 2D). Unusually, the terminal component of the 3′-UTRs of both the RH2S (566 nucleotides) and RH2M (260 nucleotides) transcripts comprised two non-coding exons (I and II, 94 and 166 nucleotides, respectively) that are interrupted by an intron (Fig. 2A). Comparison of the second half (II) of the RH2S/M 3′-UTR with the chicken and turkey genomes places this sequence downstream of exon 5b, and constitutes the first example of 3′-UTR splicing for a visual pigment gene. By contrast, the short 3′-UTR of the RH2L transcript (III, 33 nucleotides) does not appear to be spliced. All three alternatively spliced transcripts, therefore, encode RH2 pigments with distinct carboxyl termini that differ in both length and amino acid sequence, which may result in aberrant biochemical or functional characteristics of the modified opsin proteins. Indeed, when subjected to protein motif analyses, differences in functionally important palmitoylation (Maeda et al., 2010) and phosphorylation (Ohguro et al., 1993) sites between all three RH2 variants were observed, with putative palmitoylation sites being restricted to the RH2M pigment and the absence of phosphorylation sites solely in the RH2S pigment. By contrast, the RH2M and RH2L variants have a predicted nine and three sites for kinase activity, respectively (Fig. 2C). In addition, PROSITE analysis identified a putative myristoylation site (GLLQTR) in RH2L, which is absent in other avian species (Fig. 2C,D), and which may increase the membrane half-life and pigment activity of this unique opsin (Kostenis, 2002). Finally, the RH2M variant contains a VxPx motif (i.e. V-A/S-P-A) (Fig. 2C) that is important for transport of visual pigments to the outer segment of retinal photoreceptors (Mazelova et al., 2009). This motif is absent in both RH2S and RH2L isoforms, suggesting that cilial transport may be compromised.

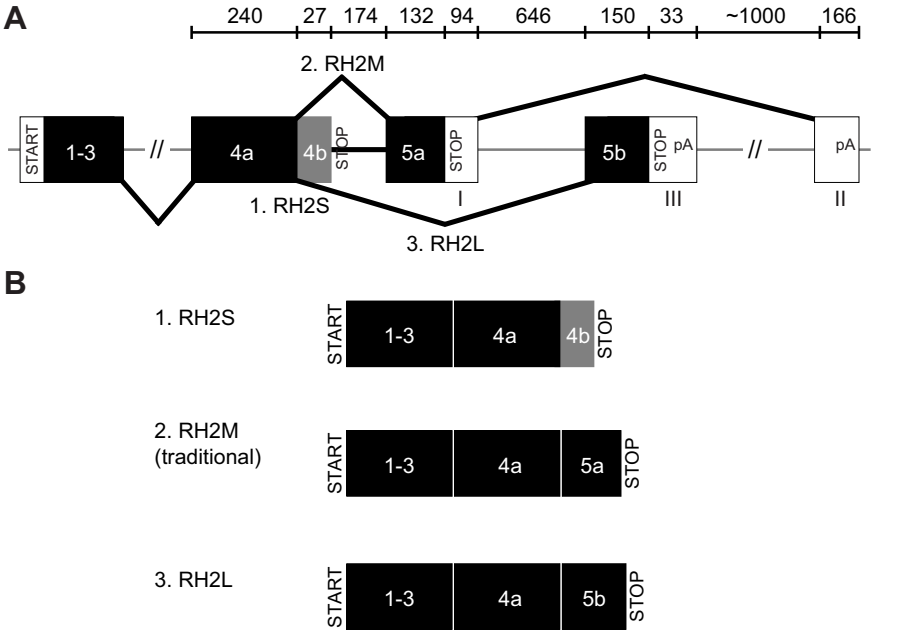

Fig. 2. Unique features of RH1 and RH2 visual pigments expressed in *Platycercus elegans*. (A) The genomic structure of the *P. elegans RH2* opsin gene (not drawn to scale), showing traditional and novel coding exons (black boxes), non-coding exons of the untranslated regions (UTRs) (white boxes) and the alternative splicing between them (black lines). START, STOP and pA refer to translation start codons (AUGs), translation termination codons (UAA, UAG, UGA) and polyadenylation signals, respectively. I, II and III denote the 3′-UTR non-coding exons discussed in the Results. Relative exon/intron sizes are shown in nucleotides. (B) A diagram showing the transcripts formed by alternative splicing of the *P. elegans RH2* opsin gene. (C) An amino acid sequence alignment of the carboxyl termini of the three spliced variants (RH2S, RH2M and RH2L) encoded by the *P. elegans RH2* opsin gene, showing the retinal binding site (K296), the boundary between exon 4 and exon 5 (vertical line), and putative palmitoylation (closed box), myristoylation (underline) and phosphorylation sites (open box). (D) Alignment of the polypeptide sequence encoded by exon 5b derived from *P. elegans*, chicken (*Gallus gallus*) and turkey (*Meleagris gallopavo*) genomes. Predicted phosphorylation (open box) and myristoylation (underline) sites are highlighted. (E) Partial sequence alignment of the carboxyl termini of RH1 pigments from an array of representative vertebrates (supplementary material Table S2), showing the extension of the rod pigment in *P. elegans*. Experimentally determined and putative protein motifs are highlighted as follows: palmitoylation (closed box); myristoylation (underline); phosphorylation (open box); cilial transport sequence, VxPx (grey box); and PDZ-binding domains, TRL and TRVL (overline). For panels C–E, conserved amino acids are shown by asterisks (*) and gaps inserted to maximise sequence identity by a hyphen (-). RH2S, RH2-short; RH2M, RH2-medium; RH2L, RH2-long (panels A–C).

Although the *P. elegans* RH1 gene generated a single mRNA transcript, the translated protein sequence possesses a unique extension of 25 amino acids at the carboxyl terminus (GPVGGLLSPALGTLGTRLQPRTRVL; Fig. 2E). The carboxyl termini of rod pigments are highly conserved across all vertebrate classes in sequence and length; the difference observed in *P. elegans* suggests that this pigment may possess altered functional properties, without negatively affecting the action of the conserved VxPx motif (Fig. 2E) and subsequent cilial transport of RH1 (and cone) pigments to the outer segment (Mazelova et al., 2009). Indeed, similar to the RH2L pigment, a putative myristoylation site (GTLGTR) was identified in *P. elegans* RH1 (Fig. 2E) that may serve to increase steady-state G protein signalling through changes to membrane clearance (Kostenis, 2002) in addition to the already-present palmitoylation sites (Fig. 2E). This extended carboxyl-terminus sequence also contained TRL and TRL-like (TRVL) motifs with Post-synaptic density 95 (Ps.d. 95), Discs large (DL), ZO-1 (PDZ)-interacting domains that have been shown to increase the trafficking of many proteins to the plasma membrane (Romero et al., 2011).

Spectral tuning *P. elegans* visual pigments

From the large database of visual pigments (wild type and site-directed mutated variants) that have been both sequenced and analysed spectrally, either by *in situ* by MSP or after *in vitro* regeneration, it is now possible to predict the λ_{max} values of a pigment from a knowledge of the amino acid residues present at certain sites in the opsin protein.

For LWS opsins, a ‘five-site’ rule dictates the spectral maxima of visual pigments that belong to this class. Specifically, the presence of S164/H181/Y261/T269/A292 (SHYTA) typically yields a pigment with a λ_{max} value at 560 nm (Yokoyama, 2000a; Davies et al., 2012). Despite the presence of SHYTA in *P. elegans*, the λ_{max} value determined by MSP was long-wavelength shifted at 567 nm (Fig. 1B), although within the average range for four other birds (563±5 nm; supplementary material Table S3), suggesting that other tuning sites may be involved.

All vertebrate non-avian SWS1 pigments that are UV sensitive, including the ancestral orthologue, possess F86, which is sufficient by itself to generate a UV-sensitive pigment with a λ_{max} value close

to 360 nm (Cowing et al., 2002; Davies et al., 2007). In birds, the role of F86 has been replaced by C90 in determining UV sensitivity (Hunt et al., 2009); the latter is present in *P. elegans* and explains the UV sensitivity of the SWS1 pigment in this parrot (Fig. 1E).

The ancestral SWS2 pigment is predicted to possess a spectral peak at 440 nm based on the presence of F46/I49/V52/T93/A164/L207/A269 (FIVTALA) (Yokoyama and Tada, 2010). In *P. elegans*, the residues present at these sites are AAVTGLA, sharing only 57% sequence identity with the ancestral SWS2 sequence. Conversely, the SWS2 pigment expressed in the pigeon (*Columba livia*) (λ_{max}=448 nm) (Kawamura et al., 1999) differs only at site 52 from that in *P. elegans*. From comparisons across 33 pigments, a V52G substitution was shown to cause a short-wavelength shift of 3 nm (Yokoyama and Tada, 2003). Overall, therefore, the predicted spectral peak of the *P. elegans* SWS2 pigment is 451 nm, a value that is identical to the average λ_{max} value for SWS2 from four avian species (supplementary material Table S3). As the MSP λ_{max} value for *P. elegans* SWS2 was determined to be 440 nm, the 11 nm discrepancy may be due to *P. elegans*-specific substitutions that produce a pigment that is spectrally more similar to the ancestral variant than to other avian orthologues. It should be noted, however, that the latter MSP value was obtained from just a single cell.

Unlike the other opsin classes, the spectral sensitivity of RH2 pigments can be difficult to predict. Given the common origin of the *RH2* (cone) and *RH1* (rod) opsin genes, the accuracy in predicting spectral peaks can be improved by using a typical RH1 pigment as a point of origin [e.g. coelacanth, *Latimeria chalumnae* (Yokoyama, 2000b)]. The tuning of both RH1 and RH2 pigments is influenced by residues at seven sites (Yokoyama, 2000a); significantly, there is only a single difference at these sites between the ancestral RH1 and RH2 sequences, an A295S substitution (Davies et al., 2007), which is known to cause a 5 nm short-wavelength shift (Lin et al., 1998). All bird RH2 pigments studied so far, including that of *P. elegans*, possess the ancestral RH2 combination of tuning residues with the addition of an E122Q substitution (supplementary material Table S3). Site-directed mutagenesis analysis of the bovine (*Bos taurus*) rod opsin pigment has demonstrated that an E122Q substitution causes a 19 nm short-wavelength shift (Nathans, 1990), which would result in a predicted spectral peak for avian RH2 pigments at 476 nm, whereas these pigments are considerably long-wavelength shifted with an average λ_{max} at 505±2 nm calculated from four bird species (supplementary material Table S3). The RH2 pigment expressed in *P. elegans* possesses a spectral peak at 509 nm (Fig. 1C), which is 33 nm more long-wavelength shifted than the calculated value (supplementary material Table S3), so it would appear that the *P. elegans* RH2 pigment, and that of birds in general, possesses non-typical tuning sites for determining the spectral peak. Unlike avian RH2 pigments, the observed spectral sensitivities of RH1 pigments in birds (average λ_{max} at 503±1 nm calculated from four bird species) are generally very similar to the predicted values from ancestral avian and non-avian species (λ_{max} at 500 nm) (supplementary material Table S3). Conversely, the RH1 pigment in *P. elegans* is long-wavelength shifted to 510 nm (Fig. 1A) despite all seven known tuning sites being conserved (supplementary material Table S3), suggesting that other presently unknown tuning sites may be involved.

DISCUSSION

Using both molecular and functional assays (i.e. MSP and *in vitro* regeneration), this study has demonstrated that five opsin genes encoding a rod and four cone visual pigments are expressed in the retina of *P. elegans adelaidae*. These opsin genes are orthologous to the pigments classes found in other birds and the vertebrates in general (Yokoyama, 2000a; Davies et al., 2012). Although visual pigments present in many avian species have been identified, only in three species [the chicken (Okano et al., 1992), the pigeon (Kawamura et al., 1999) and the zebra finch (*Taeniopygia guttata*) (Yokoyama et al., 2000)] have the full complement of rod and cone opsin genes been identified and sequenced. Our study therefore adds a further species to this list and forms the most complete record to date of the opsin genes and visual pigments present in a parrot species. It is the first to identify and characterise the genetic basis of colour vision in a parrot species.

Spectrally, the peak absorbance value for each *P. elegans* visual pigment is in broad agreement with the spectral sensitivities of other avian rod and cone photoreceptors, except for the RH1 pigment, which is long-wavelength shifted. The spectral maxima for all five *P. elegans* pigments, and those of other avian species, are, however, significantly different from the predicted λ_{max} values calculated by applying the known spectral tuning mechanisms for non-avian vertebrate visual pigments. Indeed, all five pigments peak at longer wavelengths than expected. Many vertebrates, including reptiles that clade within a sister group to the Aves, utilise a vitamin-A_2-based retinal chromophore (3,4-didehydroretinal) (Yokoyama, 2000a), resulting in the production of porphyropsin pigments that are long-wavelength-shifted compared with rhodopsins that contain a vitamin-A_1-based retinal chromophore (Whitmore and Bowmaker, 1989). MSP demonstrates that the visual pigments of *P. elegans*, as well as other birds, are rhodopsins and not porphyropsins (Hart and Hunt, 2007), a result that is consistent with the absence of 3,4-didehydroretinal in the avian visual system (Hart and Hunt, 2007). Thus, the spectral differences identified between avian and non-avian pigments are likely to be mediated by tuning mechanisms that are unique to birds. This is certainly the case for avian SWS1 photopigments that uniquely use the substitution of Ser by Cys at site 90 to shift from violet to UV sensitivity (Hunt et al., 2009), rather than changes at site 86 in non-avian vertebrate SWS1 pigments (Hunt et al., 2007); changes at this latter site exert minimal or no effects on spectral tuning in the SWS1 pigments of birds (Hunt et al., 2009).

Close inspection of the protein sequences of *P. elegans* visual opsins shows that LWS, SWS1 and SWS2 are very similar to other avian pigments, whereas both RH1 and RH2 pigments exhibit a number of unique features. Firstly, the RH1 sequence contains an extended carboxyl terminus that is longer than that seen in any other vertebrate so far studied. It should be noted, however, that only a small number of bird species have been characterised, so it is possible that such an extension may be common to all parrots, or even widespread amongst birds. Future work can test these hypotheses. The presence of this elongated carboxyl terminus is unlikely to affect cilial transport (Concepcion et al., 2002) as the VxPx motif has been retained (Mazelova et al., 2009). In addition, the extended RH1 carboxyl-terminal tail contains putative myristoylation (attachment of an acetyl group) and PDZ-binding motifs that are important for membrane trafficking, tethering and signaling of G proteins (Kostenis, 2002). These motifs may serve to increase the number of RH1 opsin molecules within the outer segment of rod photoreceptors, thereby enhancing rod photoreceptor sensitivity.

Secondly, three different RH2 mRNA transcripts are present in the retina of *P. elegans* arising from the alternative splicing of opsin and other downstream exons, as well as intron retention. The generation of multiple transcripts is known for a small number of visual opsins (al-Ubaidi et al., 1990; Applebury et al., 2000; Wistow

et al., 2002), but they are produced through the differential use of alternative polyadenylation termination signals and not by alternative splicing (al-Ubaidi et al., 1990; Applebury et al., 2000; Wistow et al., 2002). Uniquely, therefore, for cone opsin pigments, the three RH2 mRNA transcripts yield proteins with unique carboxyl termini (termed short, medium and long based on their respective lengths), a regulatory mechanism that is commonplace for some non-visual opsins such as melanopsin and VA (Davies et al., 2010), but not previously identified in the visual system. Although discovered in *P. elegans*, the novel downstream exons are conserved in other avian genomes in a location similar to that in *P. elegans*, thus suggesting that differentially spliced cone opsins may be a general property of bird RH2 opsins. The alternative splicing of RH2 transcripts results in the generation of a number of motifs that differ between the three RH2 protein sequences that might suggest these pigments exhibit distinct cellular properties. For example, sites for palmitoylation (attachment of fatty acid groups) and myristoylation are predicted for RH2M and RH2L, respectively, but are absent from RH2S. Similarly, putative serine and threonine phosphorylation sites are present in both former polypeptides, but again omitted from the short RH2 isoform. Phosphorylation sites generally cluster around the carboxyl termini of opsins (Ohguro et al., 1993), where they play an essential role in pigment deactivation and cessation of the visual phototransduction cascade (Ohguro et al., 1993). It has also been shown that the carboxyl terminus of visual opsins may directly modulate spectral sensitivity (Yokoyama et al., 2007), a process that may differentially influence the absorbance characteristics of the multiple RH2 variants expression in the *P. elegans* retina (or RH1 pigments between different avian species given the elongation of the carboxyl terminus in *P. elegans*). Therefore, it may be envisaged that different levels of expression of each RH2 variant may alter the phototransduction kinetics of the photoreceptors.

Given the propensity for red or yellow plumage coloration, or a variable intermediate in the *P. e. adelaidae* subspecies, it is possible that multiple alternatively spliced transcripts are expressed for the other four visual opsin gene classes, each with a distinct regulatory effect on vision. However, the sequences reported here were obtained from a few individuals captured in a single location of the *P. e. adelaidae* subspecies. Indeed, while the RH1 extension appears consistent across these individuals, the alternatively spliced RH2 transcripts were obtained from a single individual, so the prevalence of these unique features is currently unclear. Therefore, analyses similar to those reported here should be undertaken with additional populations and subspecies of this intriguing parrot species to determine whether these novel opsins occur in other subspecies. If so, rigorous physiological and behavioural measurements will be necessary to determine whether ocular responses and visual behaviours differ between avian species (and subspecies) that possess these features when compared with those species (or subspecies) that express the traditional opsins found in the birds studied to date. Additionally, the oil droplets located in bird retinas have a marked beneficial effect on colour resolution and colour constancy in birds (Vorobyev et al., 1998; Vorobyev, 2003; Knott et al., 2010; Knott et al., 2012), and are known to vary in response to diet (Bowmaker et al., 1993; Knott et al., 2010) and light environment (Hart et al., 2006). These oil droplets could potentially show consistent absorbance differences between *P. elegans* subspecies that could thereby cause intraspecific variation in vision. Nevertheless, it is interesting that a species exhibiting the unique and extreme plumage variation observed in *P. e. adelaidae* should also show previously unknown cone opsin transcript variation, and a rod opsin extension, thus identifying an association that warrants further study. Changes to ambient light environments are known to influence colour patterns in birds and the potential for sensory drive (Marchetti, 1993); the intraspecific visual pigment variation, generated from distinct opsin proteins with unique features that might affect spectral sensitivity and cellular photokinetics, may be linked, therefore, to the plumage variation within the *P. elegans* species complex or the Aves in general.

LIST OF SYMBOLS AND ABBREVIATIONS

MSP	microspectrophotometry
RACE	rapid amplification of cDNA ends
λ_{max}	wavelength of peak absorbance spectra

AUTHOR CONTRIBUTIONS

B.K.: opsin sequencing; all MSP data collection and analysis; preparation of manuscript. W.I.L.D.: opsin sequencing; analysis and interpretation of opsin sequences; phylogenetic analysis; *in vitro* spectral analysis; preparation of manuscript. L.S.C.: *in vitro* spectral analysis of SWS1 opsin. M.L.B.: senior investigator on *P. elegans* research projects; all fieldwork and collection of retinal samples; assisted with manuscript preparation. K.L.B.: senior investigator on *P. elegans* research projects; assisted with manuscript preparation. J.K.B.: provision of microspectrophotometer (UCL), MSP training and supervision; assisted with MSP data analysis and interpretation; assisted with manuscript preparation. A.T.D.B.: conceived, managed and oversaw the project; provided funding and equipment; principal investigator of *P. elegans* research grants; assisted with manuscript preparation. D.M.H.: interpretation of molecular sequences; provision of laboratory resources for PCR and gene sequencing; assisted with manuscript preparation. B.K. and W.I.L.D. contributed equally to this research.

COMPETING INTERESTS

No competing interests declared.

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