Ultraviolet-sensitive vision in long-lived birds

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Long-term exposure to ultraviolet (UV) light generates substantial damage, and in mammals, visual sensitivity to UV is restricted to short-lived diurnal rodents and certain marsupials. In humans, the cornea and lens absorb all UV-A and most of the terrestrial UV-B radiation, preventing the reactive and damaging shorter wavelengths from reaching the retina. This is not the case in certain species of long-lived diurnal birds, which possess UV-sensitive (UVS) visual pigments, maximally sensitive below 400 nm. The Order Psittaciformes contains some of the longest lived bird species, and the two species examined so far have been shown to possess UVS pigments. The objective of this study was to investigate the prevalence of UVS pigments across long-lived parrots, macaws and cockatoos, and therefore assess whether they need to cope with the accumulated effects of exposure to UV-A and UV-B over a long period of time. Sequences from the *SWS1* opsin gene revealed that all 14 species investigated possess a key substitution that has been shown to determine a UVS pigment. Furthermore, *in vitro* regeneration data, and lens transparency, corroborate the molecular findings of UV sensitivity. Our findings thus support the claim that the Psittaciformes are the only avian Order in which UVS pigments are ubiquitous, and indicate that these long-lived birds have UV sensitivity, despite the risks of photodamage.

Keywords: visual pigments; ultraviolet; photodamage; spectral tuning; *SWS1* opsin; parrots

1. INTRODUCTION

Photodamage arising from exposure to short wavelength light is a serious problem for numerous plants and animals, and in vertebrates, damage extends to the retina [1]. In humans, the cornea and lens absorb all ultraviolet (UV)-A (315–400 nm) and most of the terrestrial UV-B (300–315 nm) radiation; the reactive and damaging shorter wavelengths of light are thereby prevented from reaching the retina. However, the screening of shorter wavelengths is not present in species that possess UV-sensitive (UVS) photoreceptors that permit vision into the UV. Among Eutherian mammals, UV sensitivity appears to be present only in mice and rats but is more widespread among marsupials [2,3]. Most are however nocturnal, so exposure to high light levels is relatively limited, and they are all relatively short-lived, thereby allowing less time for damage to accumulate. However, this is not the case in certain species of long-lived diurnal birds.

Birds are highly dependent on vision with a visual system that contains an elaborate mechanism for colour detection [4–6], with up to four classes of visual pigment present in single cones that most probably serve tetrachromatic colour vision [4–9]. Cone photoreceptors contain

coloured oil droplets in the distal regions of their inner segments that are spectrally matched to the visual pigment and act to absorb all wavelengths shorter than a droplet-specific cut-off wavelength [10,11]; short wavelength absorption in the single cones arises from carotenoids, the concentration of which is resistant to dietary manipulation [12]. In all species, the short wavelength-sensitive visual pigment is specified by the short wavelength-sensitive type 1 (*SWS1*) gene class; the ancestral form of this gene almost certainly specified a UVS pigment with peak sensitivity (λ_{max}) below 390 nm [2]. *SWS1* pigments also exist in a violet sensitive (VS) form with $\lambda_{\text{max}} > 390$ nm, and it is a VS version of the pigment that is present in most mammals including humans. At the base of the avian lineage, UV-sensitivity was almost certainly lost with the majority of extant species now possessing VS pigments [2]. However, UVS pigments are found in 4 out of 14 avian Orders examined to date [13,14], where it has been re-acquired independently in each Order by a single amino acid substitution of Ser by Cys at site 90 [15–17]. In the four Orders containing species with UVS pigments (Passeriformes, Struthioniformes, Ciconiiformes, Psittaciformes), some species have VS rather than UVS pigments, but this is not found in the Psittaciformes where both the species (budgerigar; African grey) examined so far have UVS pigments. The molecular mechanism for the generation of UVS pigments differs in non-avian vertebrates [11], thereby reinforcing

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the conclusion that avian UVS pigments have a separate and more recent origin [17].

The use of UV waveband in reflectance-mediated cues in sexual selection, foraging and other visually guided behaviours has been extensively documented in birds [18–29]. There are, however, associated costs of UV sensitivity. UV light has a higher scattering, according to $1/\lambda^4$ (Rayleigh effect), causing chromatic aberrations that reduce spatial resolution and contrast, and hence detection of distant objects (reviewed in Bennett & Cuthill [30]). These physical obstacles can, nonetheless, be reduced by high-quality optical media and close distance to the object. More critically, UV light is damaging when absorbed by biological molecules, particularly DNA, and high levels of UV radiation are known to cause cellular damage to retinal and ocular tissue both *in situ* and *in vitro*, which mostly accumulates with age [31–34].

The presence of UVS cones in the avian retina was first demonstrated by microspectrophotometry in the Pekin robin, *Leiothrix lutea* [35] and subsequently by *in situ* hybridization in the budgerigar, *Melopsittacus undulatus* [36]. The budgerigar is a member of the Order Psittaciformes that contains some of the most long-lived birds, such as the cockatoos and macaws [37]. If UV-sensitivity is a conserved trait across the Order, this raises several issues regarding the use of UV signals, especially in relation to the effects on the retina of a life-time exposure to UV radiation. Preliminary evidence that UV-sensitivity is present has been obtained from a partial sequence of the *SWS1* gene from the African grey parrot; Cys90 is encoded [13] as present in other avian species shown to possess UVS pigments [15,16]. The main objective of this study was, therefore, to assess whether UVS pigments are widespread throughout a diverse selection of long-lived parrot species (from Australia, South America, New Zealand and Indonesia) and to determine the transparency of the lens to UV light.

2. MATERIAL AND METHODS

(a) cDNA synthesis and rapid amplification of cDNA ends

Poly(A)⁺ mRNA was extracted from *Platyercus elegans adelaidae* (rosella) retinae using the QuikPrep Micro mRNA Purification Kit (Amersham Biosciences, distributed by GE Healthcare Life Sciences, Little Chalfont, UK). Retinae had been stored in RNAlater (Ambion, Austin, TX, USA) since collection from free-living birds caught under licence in South Australia (34°57' S, 138°49' E). Single-stranded cDNA was synthesized using the 5'–3' RACE Kit 2nd Generation (Roche Diagnostics, Burgess Hill, UK). Partial *SWS1* opsin fragments were amplified using degenerate primer pairs BUD1F/BUD418R and AOASF/AOASR (electronic supplementary material, table S1). From these partial sequences, species-specific 3'- and 5'-end primers were designed to obtain the full-length coding sequence for the *P. elegans SWS1* opsin gene (GenBank accession no. HM150794) using the 3'–5' RACE Kit 2nd Generation (Roche Diagnostics).

(b) In vitro expression and regeneration of SWS1 opsin pigment

The PCR amplification of the coding sequence of the *P. elegans SWS1* opsin and subsequent cloning into the

pMT4 vector followed previously published protocols [38,39]. The PCR primers used are listed in electronic supplementary material, table S1. The expression construct containing the *P. elegans SWS1* opsin was used to transiently transfect HEK 293T cells using GeneJuice Transfection Reagent (Novagen, distributed by Merck, Nottingham, UK). Tissue culture plates of 12 × 140 cm were used and at 48 h post-transfection, the cells were harvested and washed four times with phosphate-buffered saline (PBS-pH 7.0). Cell pellets were stored at –80°C prior to regeneration of the pigment. The regeneration, solubilization and purification of the pigments followed the method of Molday & MacKenzie [40] and have been briefly described in Carvalho *et al.* [17].

(c) Determination of $\lambda_{\max}$ of expressed pigments

The absorbance spectrum of the purified pigment (dark spectrum) was determined by UV-vis spectroscopy using a Spectronic Unicam UV500 dual-beam spectrophotometer by scanning from 250 to 600 nm. The sample was then treated with hydrochloric acid (1 M) to denature the protein and re-scanned. This acid-treated spectrum was then subtracted from the dark spectrum to give a difference spectrum, which thereby avoids distortion caused by underlying absorbance and scatter by the protein. This was then used to determine the $\lambda_{\max}$ by fitting a standard visual pigment template [41] using a Solver add-in to Microsoft EXCEL, which varies the $\lambda_{\max}$ until the best fit to the template is found.

(d) Lens transmission analysis

The transmission of *P. elegans* lens was measured by scanning spectrophotometry from 300 to 700 nm [42].

(e) Amplification and partial sequencing of the SWS1 opsin gene

Retinal cDNAs from four endemic Australian parrot species were kindly donated by Prof. Lyn Beazley (University of Western Australia, Perth, Australia) and include the twenty eight parrot (*Barnardius zonarius semitorquatus*), sulphur-crested cockatoo (*Cacatua galerita*), Carnaby's black cockatoo (*Calyptrorhynchus latirostris*) and the galah (*Eolophus roseicapilla*) (table 1). From the full-length sequence obtained for the *P. elegans SWS1* opsin, gene-specific primers ROSELLA SWS1 F/ROSELLA SWS1 R (electronic supplementary material, table S1) were designed to the 5'- and 3'-end of the coding sequence. PCR amplification generated a product of around 1 kb for all four species of Australian parrots. These products were then cloned and fully sequenced. Genomic DNA for the remaining parrot species (table 1) was extracted from plucked blood feathers stored at –20°C. Partial *SWS1* opsin gene sequences were obtained using primer pair BUD1F/BUD418R (electronic supplementary material, table S1), generating a fragment of around 1.8 kb encoding the complete sequence of exon 1 and intron 1 and a partial sequence of exon 2. PCR fragments were then cloned and fully sequenced.

(f) Gene tree

Neighbour-joining [43] was used to construct a gene tree from opsin nucleotide sequences after alignment with CLUSTAL X [44]. The degree of support for internal branching was assessed by bootstrapping with 1000 replicates using the MEGA2 computer package [45].

Table 1. List of the parrot species from which the *SWS1* opsin gene sequence was investigated in this study.

scientific name	common name	family	geographical location	DNA type	accession numbers
<i>Amazona versicolor</i>	St Lucia amazon	Psittacidae	Caribbean	gDNA	HM150793
<i>Amazona guildingii</i>	St Vincent amazon	Psittacidae	Caribbean	gDNA	HM150795, HM150796
<i>Anodorhynchus hyacinthinus</i>	hyacinth macaw	Psittacidae	South America	gDNA	HM150806
<i>Ara ararauna</i>	blue and gold macaw	Psittacidae	South America	gDNA	HM150803
<i>Ara chloroptera</i>	green wing macaw	Psittacidae	South America	gDNA	HM150805
<i>Ara macao</i>	scarlet macaw	Psittacidae	South America	gDNA	HM150792
<i>Cacatua moluccensis</i>	Moluccan cockatoo	Cacatuidae	Indonesia	gDNA	HM150797, HM150798
<i>Barnardius zonarius</i>	twenty eight parrot	Psittacidae	Australia	cDNA	HM150799
<i>semitorquatus</i>					
<i>Cacatua galerita</i>	sulphur-crested cockatoo	Cacatuidae	Australia	cDNA	HM150802
<i>Calyptorhynchus latirostris</i>	Carnaby's black cockatoo	Cacatuidae	Australia	cDNA	HM150800
<i>Eolophus roseicapillus</i>	Galah	Cacatuidae	Australia	cDNA	HM150801
<i>Platycercus elegans</i>	crimson rosella	Psittacidae	Australia	g/cDNA	HM150794
<i>Nestor notabilis</i>	Kea	Nestoridae	New Zealand	gDNA	HM150807
<i>Psittacus erithacus</i>	African grey parrot	Psittacidae	Africa	gDNA	HM150804

3. RESULTS

(a) *Platycercus elegans* SWS1 opsin

The full-length coding sequence of the *P. elegans* SWS1 gene plus the 5'- and 3'-UTR regions has been deposited in GenBank (accession no. HM150794). Figure 1*a* shows the predicted amino acid sequence aligned with the budgerigar, pigeon (*Columba livia*) and chicken (*Gallus gallus*) SWS1 opsins. When compared with these other sequences, the *P. elegans* SWS1 sequence shows 94, 90 and 84 per cent nucleotide identity and 97, 90 and 84 per cent amino acid identity, respectively, to the budgerigar, pigeon and chicken SWS1 sequences. Figure 1*b* shows the phylogenetic relationships among the avian SWS1 opsin genes, placing the *P. elegans* sequence in close proximity to the budgerigar SWS1 gene and is consistent with the close evolutionary relationship of these two species.

(b) Spectral sensitivity of the *Platycercus elegans* SWS1 pigment

Inspection of the *P. elegans* sequence shows that the residue present at site 90 is a Cys (bovine opsin numbering), the same residue found in the budgerigar UVS SWS1 opsin (figure 1*a*) and shown by Wilkie *et al.* [15] to be responsible for UVS. In order to confirm that the *P. elegans* SWS1 pigment is also UVS, the full-length wild-type coding sequence was expressed *in vitro*; the resulting pigment yielded a $\lambda_{\max}$ value in the UV at 363 nm (figure 2).

(c) Transmission of the *Platycercus elegans* lens

The spectrum of light transmission of the *P. elegans* lens is shown in figure 3. Two lenses were analysed and both showed around 70 per cent transmission at 350 nm, indicating that the *P. elegans* lens is transparent to UV light.

(d) Partial sequences of the SWS1 opsin gene

A further 13 species were studied as listed in table 1. These included representative species of South American

macaws, Caribbean amazons, Indonesian and Australian cockatoos, and Australian broad-tailed parrots, plus the African grey parrot and the New Zealand Kea. Partial SWS1 opsin sequences of 393 bp of coding sequence that encompasses exons 1–2 were obtained from either cDNA or gDNA. The starting point for these partial fragments is at nucleotide +25 downstream of the translation start codon and extends to nucleotide position 417 in exon 2. The exon 1/2 boundary is located between nucleotide positions 346 and 347. Of the sequences obtained here, part of the African grey parrot SWS1 gene sequence has been previously reported (GenBank accession no. AY227186) by Odeen & Hastad [13], although this sequence extends only from nucleotide positions 219 to 278 in exon 1. The translated nucleotide alignment for these partial sequences is shown in figure 4. In all species, Cys90 is present and this is combined with Ala86 as found in the budgerigar SWS1 pigment [15].

4. DISCUSSION

The sequences of the SWS1 opsin gene presented here for a total of 14 Neotropical, Australasian and African species sampled across the Psittacidae, Cacatuidae and Nestoridae families, show that all have Cys90. Previous studies by Wilkie *et al.* [15] and Yokoyama *et al.* [16] have shown that in avian pigments, the presence of Cys90 confers UV sensitivity, whereas VS pigments from other species have Ser90. Furthermore, the *in vitro*-expressed rosella SWS1 pigment gave a peak at 363 nm, thereby confirming the presence of UVS pigments in the larger and longer lived parrot species. The Psittaciformes are therefore the only avian order where all species studied to date have a UVS pigment and is strong evidence that the Ser90Cys substitution responsible for this peak shift into the UV occurred at the base of the Psittaciformes lineage, and has been retained in all extant species.

Consistent with the presence of UVS pigments, the lens of *P. elegans* has a 50 per cent transparency cut-off at around 320 nm, which would allow a substantial

Figure 1. (a) *Platycercus elegans* SWS1 protein sequence aligned with budgerigar (GenBank accession no. Y11787), chicken (GenBank accession no. M92039) and pigeon SWS1 (GenBank accession no. AF149234) sequences. Residue 90, Glu113 and Lys296 are labelled. (b) Tree of full-length coding sequences of avian SWS1 opsin genes, showing the correct positioning of the rosella sequence among the avian SWS1 sequences. GenBank accession no.: pigeon SWS1 AH007798; canary SWS1 AJ277922; zebra finch SWS1 NM_001076704; cormorant SWS1 EF568933; chicken SWS2 M92037; chicken Rh1 NM_205490; chicken Rh2 M92038; chicken LWS NM_205409. The tree was generated by neighbour-joining using the Kimura-2 parameter model. The robustness of each branch point is indicated by the bootstrap values. The scale bar indicates the number of nucleotide substitutions per site. The tree was rooted by using the chicken opsins as outgroups.

proportion of UV light to reach the retina. This rises to 76 per cent transmission at the peak sensitivity of 363 nm of the expressed pigment. Coloured oil droplets that act as cut-off filters are routinely found in avian single cones. The cut-off wavelengths of these droplets are matched to the spectral sensitivity of the different cone pigments [12,46] and would therefore offer some protection to photoreceptor outer segments. UVS cones however have T-type (transparent) droplets that show no detectable absorbance from 330 to 800 nm, so these droplets would not significantly reduce the penetration of UV

light into the outer segments of UVS photoreceptors. Nevertheless, all photoreceptor cells would be exposed to transmitted UV light proximal to the oil droplets; thus, the need for photoprotection for the nucleus and other organelles proximal to the oil droplets might be similar for all photoreceptor types, and indeed for many cell types in the retina. Whether long-lived birds compared with short-lived birds have increased lipopigment in the retinal pigment epithelium, or better mechanisms for removal of peroxidized lipids in the outer segments, remains to be determined.

Figure 2. *Platyserus elegans* wild-type *SWS1* opsin pigment regenerated *in vitro* with 11-*cis*-retinal and lens transmission. (a) Raw data for the dark- (black circles) and acid-treated (grey triangles) spectra. (b) Difference spectrum (symbols) fitted with the Govardovskii visual pigment standard curve (black line) to give an accurate $\lambda_{\max}$.

Figure 3. *Platyserus elegans* lens transmission. Right (grey triangles) and left (black circles) eye lenses transparency properties measured by the transmission percentage plotted against wavelength.

The selective pressures that were responsible for the re-acquisition of UVS pigments in birds are unclear. The *SWS1* opsin sequences for the 14 parrot species reported here are highly conserved: when compared with the budgerigar sequence, only a small number of nucleotide differences are observed, with very few of these differences being non-synonymous. A better understanding of the visual ecology of these birds will be essential in determining how UV sensitivity is employed. UV-reflecting plumages are found ubiquitously across all avian lineages, including parrots, suggesting that UV reflectance is an ancestral characteristic of the avian order [27,47,48]. The highest percentage (more than 10%) of UV reflectance is usually found in blue and white feathers, although feathers reflecting predominantly at longer wavelengths can have a biologically significant UV reflectance [28,47–49]. Parrots have the highest percentage of UV reflectance, especially in plumage patches

Figure 4. Amino acid sequences deduced from the partial *SWS1* amplicons from 14 parrot species aligned with the corresponding region of the budgerigar sequence. Note that site 86 is occupied by Ala and site 90 by Cys in all species. The transmembrane regions 1–3 are boxed.

when compared with all other avian orders, and in a survey of 143 species, all but three were shown to possess a considerable amount of UV-reflecting plumage [48].

With the exception of *P. elegans* that has an expected lifespan of around 15–20 years in captivity, all the other species investigated here are considered long-lived with lifespan of up to 50 years or more in captivity [37]. UV radiation is known to damage several components of the eye such as the cornea, lens, the retinal pigment epithelium and the photoreceptors, leading to apoptosis in the cornea and pigment epithelium [33], decreased lens cell viability [50,51], the induction of oxidative stress [52] and the inactivation of anti-oxidant enzymes like catalase [34]. UV radiation is also associated with several age-related conditions like cataract and macular degeneration [33,50]. In mammals, a long lifespan would appear to be associated with a loss of UV-sensitivity of the visual system with the peak sensitivities of the *SWS1* pigments

shifted to wavelengths greater than 400 nm [11]. Only short-lived species such as the rat and mouse among the Eutheria have retained UVS pigments although, from the species studied so far, this may be more prevalent among the metatheria [3,11]. The penetration of UV radiation in the neural retina in long-lived parrot species might be expected therefore to cause irreversible damage [34,52] and raises the question as to how these species tolerate UV exposure. One explanation may be that birds show a lower rate of mitochondrial free-radical production in comparison with mammals [53,54]. Furthermore, cells of long-lived birds exhibit exceptional resistance to oxidative-induced damage when compared with shorter-lived birds and mammals [55], although the underlying genetic and molecular basis for this higher oxidative resistance is unknown. A third explanation could be the presence of carotenoids, which are present not only in the oil droplets of the retina, but also in the serum of parrots at concentrations comparable to that in other bird species [12,56]. Carotenoids are known to be effective antioxidants and immunostimulants in birds (reviewed in [57]), with lutein and zeaxanthin being especially effective in conferring protection against oxidative damage in the retina [58,59] and light-induced photoreceptor apoptosis [60]. Similarly, psittacofulvin pigments, found so far only in parrot feathers, are thought to have considerable antioxidant properties [61–63], and could offer protection if also present in the retina.

If such processes are occurring in the retina, the combination of a lower production of free radicals and an increased resistance to oxidative damage could protect the eye against the damaging effects of UV light exposure in long-lived parrot species. A more detailed study of these potential protection mechanisms could provide valuable information on the survival of retinal cells. Such studies would reveal not only how extant long-lived diurnal terrestrial birds avoid UV-induced photodamage, but may also give new insights into UV protection, thereby facilitating improvements in human health associated with reducing or ameliorating UV-induced photodamage.

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