Review

Evolution and spectral tuning of visual pigments in birds and mammals

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Variation in the types and spectral characteristics of visual pigments is a common mechanism for the adaptation of the vertebrate visual system to prevailing light conditions. The extent of this diversity in mammals and birds is discussed in detail in this review, alongside an in-depth consideration of the molecular changes involved. In mammals, a nocturnal stage in early evolution is thought to underlie the reduction in the number of classes of cone visual pigment genes from four to only two, with the secondary loss of one of these genes in many monochromatic nocturnal and marine species. The trichromacy seen in many primates arises from either a polymorphism or duplication of one of these genes. In contrast, birds have retained the four ancestral cone visual pigment genes, with a generally conserved expression in either single or double cone classes. The loss of sensitivity to ultraviolet (UV) irradiation is a feature of both mammalian and avian visual evolution, with UV sensitivity retained among mammals by only a subset of rodents and marsupials. Where it is found in birds, it is not ancestral but newly acquired.

Keywords: opsins; visual pigments; spectral tuning; evolution

Visual pigments are based on an opsin protein that is covalently linked via a Schiff base (SB) to the chromophore retinal. Five classes are found in the photoreceptors of vertebrates—a rod or Rh1 class restricted to rod photoreceptors and four different cone classes distinguished on the basis of the spectral sensitivity ($\lambda_{\max}$) and amino acid sequence of their respective opsins: longwave-sensitive (LWS) with $\lambda_{\max}$ 500–570 nm, middlewave-sensitive (MWS) with $\lambda_{\max}$ 480–530 nm and two shortwave-sensitive classes, SWS2 with $\lambda_{\max}$ 400–470 nm and SWS1 with $\lambda_{\max}$ 355–445 nm. In all classes, the spectral sensitivity of the pigment arises from interactions between the chromophore and the amino acid residues that form the retinal-binding pocket of the opsin. The visual pigments of all birds and mammals are rhodopsins as they use 11-*cis*-retinal. In vertebrate visual pigments with $\lambda_{\max}$ values greater than 385 nm, the SB is protonated, with a negatively charged residue at site 113 (usually Glu113) acting as a counterion to stabilize the proton of the SB.

1. PHOTORECEPTORS, COLOUR VISION AND THE EARLY EVOLUTION OF VISUAL PIGMENTS

Rod photoreceptors function in dim light vision, whereas cones give vision in daylight and the potential

for colour vision. The minimum requirement for colour vision is two spectrally distinct classes of cone photoreceptors, each containing a different class of visual pigment. In many vertebrates, however, the spectral sensitivity of cone photoreceptors may be modified by the presence of oil droplets that are located in the distal region of the inner segment of cone photoreceptors and act as cut-off filters. Coloured oil droplets are ubiquitous in reptiles and birds, retained as colourless droplets in marsupials (metatherians) and monotremes, but completely absent from all eutherian mammals. This absence has been attributed to a nocturnal phase that is thought to characterize the early evolution of mammals (Walls 1942).

Phylogenetic analysis based on gene sequence identity shows that the evolution of cone pigments preceded the rod pigment (Okano *et al.* 1992) (figure 1). Cone opsin sequences show an overall identity of around 40 per cent. In contrast, the MWS (or Rh2) cone and rod (Rh1) opsins show a much higher identity of around 80 per cent, indicating a more recent separation of the *Rh1* and *Rh2* gene lineages and consistent with the origin of the rod opsin gene from an ancestral duplication of the *Rh2* cone opsin gene.

2. MAMMALS

The relative frequency of rods and cones in the mammalian retina varies considerably. Most mammals have a rod-dominated retina; nocturnal species have up to 3 per cent cones and diurnal mammals between 5 and 30 per cent cones (Peichl 2005). Only in a few species

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Figure 1. Retention and loss of rod and cone opsin classes in mammals. The origin of the L and M variants of the *LWS* gene in Old World primates and one species of New World primate by gene duplication is shown.

does the proportion of cones exceed 50 per cent, with approximately 85 per cent in the ground squirrel, *Spermophilus beecheyi* (Kryger *et al.* 1998) and 95 per cent in the tree shrew, *Tupaia glis* (Muller & Peichl 1989).

(a) Evolution of cone pigments

A nocturnal phase that is thought to mark the early evolution of the mammals around 150–200 Ma may be the cause of the reduction in the number of cone pigment genes to only two classes (figure 1). In marsupial and eutherian mammals, the *LWS* gene is paired with the *SWS1* gene, with the loss of the *SWS2* and *Rh2* genes. The peak sensitivities of the *SWS1* pigments range from the ultraviolet (UV) at 360 nm as found, for example, in the mouse *Mus musculus* (Yokoyama *et al.* 1998) to as long as 440 nm as found in the tree squirrel *Sciurus carolinensis* (Carvalho *et al.* 2006). The *LWS* pigment shows less variability, ranging in most species from 530 to 565 nm, although in the mouse it is blue-shifted to 508 nm (Sun *et al.* 1997).

In contrast, the egg-laying protherian mammals, the platypus, *Ornithorhynchus anatinus*, and echidna, *Tachyglossus aculeatus*, belonging to the order Monotremata that diverged from the marsupial/placental mammal lineage around 200 Ma, have discarded the *SWS1* gene but retained the *SWS2* gene along with the *LWS* gene (Davies *et al.* 2007; Wakefield *et al.* 2008). The spectral peaks of the corresponding pigments of the platypus are at 451 and 550 nm, respectively. The *LWS* peak at 550 nm is similar to that found for *LWS* pigments in many marsupial and eutherian mammals, and the *SWS2* peak at 451 nm is at only a marginally longer wavelength than the violet-sensitive (VS) *SWS1* pigments of some placental mammals. The dichromacy of the platypus is not dissimilar, therefore, to that of the tree squirrel with an *SWS1* pigment peaking at 440 nm (Carvalho *et al.* 2006). The presence of the *SWS2* gene in monotremes also means that ancestral mammals prior to the protherian/therian split must have retained both *SWS* genes, which, in combination with the *LWS* gene, would have provided the basis for trichromacy.

With the exception of primates (discussed subsequently), eutherians are at best dichromats with S and L cones that contain, respectively, SWS1 and LWS pigments. There is, however, evidence that some Australian marsupials have a class of M cones in addition to S and L cones, which provide for trichromacy (Arrese *et al.* 2006). This was first reported for the fat-tailed dunnart, *Sminthopsis crassicaudata*, and the honey possum, *Tarsipes rostratus* (Arrese *et al.* 2002), and subsequently extended to the quokka, *Setonix brachyurus*, and quenda or bandicoot, *Isodon obesulus* (Arrese *et al.* 2005). Attempts, however, to identify this third cone pigment have failed, despite extensive efforts in two laboratories (Strachan *et al.* 2004; Cowing *et al.* 2008). A second rod *Rh1* pigment gene was however identified in the genome of the fat-tailed dunnart by Cowing *et al.* (2008), and these authors have advanced the hypothesis that M cones express a rod pigment.

(b) Trichromacy in primates

Primates comprise the two major simian groups: the New World (platyrrhine) primates from Central and South America and the Old World (catarrhine) primates from Africa and Asia, plus the prosimians (lorises, lemurs and the tarsiers). Trichromacy is found throughout all three groups (Nathans *et al.* 1986; Bowmaker *et al.* 1991; Ibbotson *et al.* 1992), with the major evolutionary driving force being an improved colour discrimination in the red/green region of the spectrum in the detection and evaluation of ripe fruits (Mollon 1989; Osorio & Vorobyev 1996; Sumner & Mollon 2000; Regan *et al.* 2001) or young nutritious leaves (Dominy & Lucas 2001) against the green foliage of the rainforest. This topic is discussed in more detail in Jacobs (2009). The evolutionary mechanism underlying this trichromacy differs, however, between the two major simian groups, with the prosimians following the platyrrhine model. In catarrhines (which include the Old World monkeys and great apes), trichromacy arises from the combination of an autosomal *SWS1* gene and duplicated copies of the *LWS* gene, one of which encodes an MWS (M) pigment with $\lambda_{\max}$ around 535 nm and the other an LWS (L) pigment with $\lambda_{\max}$ around 563 nm. The *LWS* gene duplication occurred at the base of the catarrhine lineage (Nathans *et al.* 1986) to form an opsin gene array on the X chromosome with an upstream L gene and one or more M genes downstream (Nathans *et al.* 1986; Drummond-Borg *et al.* 1989; Feil *et al.* 1990).

In platyrrhines and prosimians, the autosomal *SWS1* gene is paired with only a single copy of the X-linked *LWS* gene. This gene is, however, polymorphic (Neitz *et al.* 1991; Williams *et al.* 1992) in most platyrrhine species, with the different allelic copies that specify pigments with $\lambda_{\max}$ values ranging from 535 to 565 nm (table 1). As only females have two X chromosomes, full trichromacy is limited to heterozygous females with different allelic forms of the gene. Homozygous females as well as all males are therefore dichromats (Mollon *et al.* 1984). A polymorphic *LWS* gene is also found in three diurnal

Cetaceans are closely related to the Artiodactyla, but have a quite separate evolutionary origin from the Carnivora, from which the Pinnipedia are derived. Both groups have, however, lost a functional *SWS1* gene and lack S cones (Peichl & Moutairou 1998; Levenson & Dizon 2003; Newman & Robinson 2005; Levenson *et al.* 2006). These species have, therefore, lost colour vision, retaining only an LWS gene and L cones alongside rod photoreceptors. For those species in which molecular analyses have been carried out, the *SWS1* gene is still recognizable in the genome as a pseudogene with numerous deleterious mutations (Levenson & Dizon 2003; Newman & Robinson 2005; Levenson *et al.* 2006). It would appear likely that the dimly lit habitat of the deep ocean frequented by these aquatic mammals resulted in the relaxation of the pressure to maintain colour vision, leading to the accumulation of mutations in the *SWS1* gene. The retention of the LWS pigment in preference to the SWS1 pigment may reflect the relative paucity of S cones compared with L cones in the mammalian retina.

The Sirenia form the third group of aquatic mammals, represented by the manatees. As members of the Mesaxonia, their closest terrestrial relatives are the elephants. Manatees have been shown to be dichromats (Cohen *et al.* 1982; Griebel & Schmid 1996) with SWS1 and LWS pigments (Newman & Robinson 2006), consistent with their more shallow water habitat of coastal and estuarine areas where they feed on submerged grasses.

(d) Nocturnal and fossorial adaptations

Although many mammals are nocturnal, both SWS1 and LWS cone pigments are frequently retained in a rod-dominant retina. However, loss of colour vision is not unexpected in nocturnal species, in which activity occurs at light levels largely below the sensitivity of cones. L cone monochromacy is found in two nocturnal carnivores: the raccoon, *Procyon lotor* and kinkajou, *Potos flavus* (Jacobs & Deegan 1992). Among the rodents, most members of the squirrel family are strictly diurnal with cone-rich retinæ (Long & Fisher 1983; Blakeslee *et al.* 1988; Kryger *et al.* 1998). However, flying squirrels are nocturnal and possess an *SWS1* pseudogene with multiple deletions (Carvalho *et al.* 2006). Flying squirrels have therefore dispensed with colour vision, and this may be associated with the switch from the diurnality of their immediate tree squirrel ancestor. Many other rodents are nocturnal, but most appear to have retained two functional cone pigment genes. Examples are the mouse, rat, pocket gopher, gerbil, cururo and degu (Jacobs *et al.* 1979, 2003; Govardovskii *et al.* 1992; Jacobs & Deegan 1994a; Chavez *et al.* 2003; Peichl *et al.* 2005; Williams *et al.* 2005). The situation in Syrian hamsters is a little less straightforward as S and L cones are found in the Siberian dwarf hamster, *Phodopus sungorus*, whereas S cones are lacking in the Syrian golden hamster, *Mesocricetus auratus* (Calderone & Jacobs 1999).

The loss of S cones and a functional *SWS1* gene has occurred in the subterranean (fossorial) muroid blind

mole rat (*Spalax ehrenbergi*) (David-Gray *et al.* 2002). Although the eyes of *Spalax* are atrophied and located below a layer of skin and fur embedded in a hypertrophied Harderian gland (Sanyal *et al.* 1990; Cernuda-Cernuda *et al.* 2002), both rod and LWS cone pigments are present (David-Gray *et al.* 1998; Janssen *et al.* 2000). These animals lack any ability to respond to visual images (Haim *et al.* 1983; Rado *et al.* 1992; Cooper *et al.* 1993a,b), but retain the ability to entrain locomotor behaviour to diurnal changes in environmental light (Rado & Terkel 1989; Rado *et al.* 1991; David-Gray *et al.* 1998), and removal of the eyes obliterates this response (Pevet *et al.* 1984; Rado & Terkel 1989; Rado *et al.* 1991; David-Gray *et al.* 1998). Not all subterranean rodents, however, have lost S cones. Both cone classes are present in the retinæ of the European mole, *Talpa europaea*, a subterranean insectivore with small eyes, in the bathyergid mole rats, *Cryptomys anseelli*, *Cryptomys mehowi* and *Heterocephalus glaber*, where, unusually, S cones substantially outnumber L cones (Peichl *et al.* 2004), and in the Chilean cururo, *Spalacopus cyanus*, where S cones constitute up to 20 per cent of all cones in the ventral retina (Peichl *et al.* 2005).

The two major groups of bats, the Megachiroptera and Microchiroptera, differ in their diurnal activity. The former are crepuscular (primarily active at dawn and dusk) with a well-developed visual system, whereas the latter are nocturnal and rely on acoustic orientation or echolocation more than vision. It is not surprising therefore to find that most megabats have retained S and L cones with the corresponding pigments (Wang *et al.* 2004), although there appears to be exceptions, with three species, *Rousettus madagascariensis*, *Eidolon dupreanum* and *Epomophorus gambianus*, having L cones but no S cones (Muller *et al.* 2007). The unexpected finding is that the nocturnal microbats (Wang *et al.* 2004) also express both SWS1 and LWS pigments. Moreover, the SWS1 pigments in both groups most probably peak in the UV.

The owl monkey, *Aotus*, a platyrrhine primate, is classically described as nocturnal (Levenson *et al.* 2007), lacks S cones (Wikler & Rakic 1990) and has an *SWS1* pseudogene with deleterious mutations (Jacobs *et al.* 1996b). *SWS1* pseudogenes are also present in many species of prosimians, notably in all members of the Lorisiformes, and in some species of the Cheirogaleidae family, which forms part of the Lemuriformes group (Kawamura & Kubotera 2004; Tan *et al.* 2005). The latter includes the fat-tailed dwarf lemur (*Cheirogaleus adipicaudatus*) and greater dwarf lemur (*C. major*), but their close relatives the grey lemur (*Microcebus murinus*) and Coquerel's mouse lemur (*Mirza coquereli*) have both retained a functional gene.

SWS1 and LWS pigments have also been retained in a strictly nocturnal and highly endangered primate endemic only to Madagascar, the aye-aye (*Daubentonia madagascariensis*). In this species, the opsin gene would appear to be fully functional with evidence for purifying or stabilizing selection to reduce genetic variation (Perry *et al.* 2007), indicating that dichromacy remains advantageous for the aye-aye despite their nocturnal activity pattern.

Figure 3. Absorbance difference spectra for *in vitro* regenerated wild-type and mutant SWS1 pigments. Note that substitution at site 86 is sufficient to shift the bovine VS pigment into the UV (a) and the UVS goldfish pigment into the violet region (b). Adapted from Cowing *et al.* (2002b).

(e) Spectral tuning of rod pigments

The $\lambda_{\max}$ values for rod pigments at around 500 nm are largely invariant in terrestrial mammals. One exception, however, is found as an adaptation to the 'blue' environment of the ocean encountered by the relatively deep-diving bottlenose dolphin. In this species, the $\lambda_{\max}$ is blue-shifted to 488 nm (Fasick *et al.* 1998; Fasick & Robinson 2000) as the result of amino acid substitutions at sites 83, with Asn replacing Asp, and 292, with Ser replacing Ala (note that residue numbering for all opsins follows that for bovine rod opsin) (figure 2). In other aquatic mammals that forage much closer to the surface, Asp83 and Ala292 are present, identical to the rod pigments of terrestrial mammals.

(f) Spectral tuning of cone pigments

(i) SWS1 pigments

One of the major evolutionary changes that has occurred in the evolution of the vertebrate visual system is the switch from a peak sensitivity of the SWS1 pigment in the UV to longer wavelengths in the violet region of the spectrum (Hunt *et al.* 2007). The selective pressures for this to occur may be related to the protection of the retina from the damaging effect of UV light and an improvement in the image quality on the retina.

Among eutherian mammals, ancestral UV sensitivity of SWS1 pigments has been retained by certain species, although VS pigments are more common (Hunt *et al.* 2007). The residue changes responsible for these red shifts were initially elucidated from comparisons between the amino acid sequences of UV-sensitive (UVS) pigments in the goldfish, *Carassius auratus*, and common house mouse, *M. musculus*, and the VS pigments of the cow, *Bos taurus*, and the pig, *Sus domesticus*, the latter two species from the order Artiodactyla. A common feature was the presence of Phe at site 86 in UVS pigments and its replacement by Tyr in VS

pigments (Cowing *et al.* 2002b; Fasick *et al.* 2002). Site-directed mutagenesis was then used to confirm the role of this single substitution in shifting the peak sensitivity from UV to violet (figure 3). With the single exception of primates (discussed subsequently), this single change underlies all of the changes from UV to violet sensitivity, which has occurred in the evolution of vertebrate vision (Hunt *et al.* 2007). Its role in the shift from UVS to VS pigments in mammals is displayed in figure 4.

Tyr86 is also found in the VS pigment of the aquatic West Indian manatee, *Trichechus manatus* (Newman & Robinson 2006), but not in the VS pigments of the elephant (*Loxodonta africana* and *Elephas maximus*). Ser86 is present in these species and shown by site-directed mutagenesis to be responsible for the red shift into the violet (Yokoyama *et al.* 2005). The acquisition of Ser86 in elephants must have occurred therefore within the Proboscidea lineage, following a prior Phe86Tyr substitution that occurred in the common ancestor to the Artiodactyla (figure 4).

The order Rodentia comprises two suborders: the Sciurognathi and the Hystricognathi. Among the Sciurognathi, both mouse and rat have UVS pigments as does the Chilean degu, *Octodon degus* (Jacobs *et al.* 2003) and the Siberian dwarf hamster, *P. sungorus* (Calderone & Jacobs 1999). VS pigments are, however, found in the grey (*S. carolinensis*) and ground squirrel (*Spermophilus* spp.) (Jacobs & Yolton 1969; Jacobs 1976; Jacobs & Neitz 1984), and the red shift in the grey squirrel is again achieved via a Phe86Tyr substitution (Carvalho *et al.* 2006) to a peak at 440 nm, the most red-shifted SWS1 pigment so far recorded. Tree and ground squirrels are strictly diurnal, whereas, as discussed earlier, the closely related flying squirrels are nocturnal and have non-functional SWS1 genes. Examination of these pseudogenes reveals that they too have Tyr86 (Carvalho *et al.* 2006), which means that without the deleterious mutations, they would encode VS pigments. Members of the Hystricognathi also show a mixture of UVS and VS pigments. The Chilean degu, *O. degus*, has a UVS pigment (Jacobs *et al.* 2003), whereas a VS pigment is present in the guinea pig, *Cavia porcellus* (Jacobs & Deegan 1994b). The latter pigment is unusual in having Val86 (Parry *et al.* 2004), but its replacement with Phe is sufficient to shortwave-shift the $\lambda_{\max}$ of the pigment into the UV, confirming its role in spectral tuning.

Marsupials are divided into two major orders: the Diprotodontia and the Polyprotodontia. Among species from the former order, the Tammar wallaby (*Macropus eugenii*) and quokka (*S. brachyurus*) have VS pigments with Tyr86 (Deeb *et al.* 2003; Arrese *et al.* 2005), whereas microspectrophotometry indicates that the honey possum (*T. rostratus*) has a UVS pigment (Arrese *et al.* 2002), with Phe86 retained (Cowing *et al.* 2008). Among the Polyprotodontia, UVS pigments have been confirmed by *in vitro* expression in the fat-tailed dunnart, *S. crassicaudata* (Cowing *et al.* 2008), and in the South American big-eared opossum, *Didelphis aurita* (Hunt *et al.* 2009). Phe86 is retained in both species and in another polyprotodont, the quenda (*I. obesulus*) (Arrese *et al.*

Figure 4. Mammalian phylogeny showing the presence of UVS and VS SWS1 pigments. Violet lines indicate the retention of UV sensitivity and blue lines a shift to violet sensitivity. The evolutionary position of substitutions at site 86 is indicated by their placement on the tree. Note that except for the primates, the retention of Phe86 is always associated with the retention of UV sensitivity. Dagger symbol denotes pseudogene.

Table 2. Residues present at seven sites in transmembrane helices II and III of SWS1 pigments. The sites were identified by site-directed mutagenesis as necessary to shift the λ_{max} of the human pigment from violet to UV (Yokoyama 2002).

simian					prosimian			
sites	mouse	great apes	Old World monkeys	New World monkeys	tarsiers	aye-aye	lemurs	galagos and lorises
46	F	T	T	I	V	F	L/F	F
49	F	L	L	L	S	F	F	F
52	T	F	F	L	I	T	A/T	T
86	F	L	L	L	L	F	C/S/L/V	Y/N/C
93	T	P	P	P	P	P	P	P
114	A	G	G/A	G/A	G	G	G	G
118	S	T	T	T	S	S	S/C	S
Haplorrhini					Strepsirrhini			

2005). The Phe86Tyr substitution seen in the Tammar wallaby and quokka must have occurred therefore within the diprotodont marsupial lineage and represents convergent evolution with the identical substitutions in the Eutheria. Spectral data for S cones and sequence data for SWS1 pigments in carnivores are extremely limited.

VS cones have been found in the ferret, *Mustela putorius furo* (Calderone & Jacobs 2003), spotted hyena *Crocuta crocuta* (Calderone *et al.* 2003), polar bear and sea otter (Levenson *et al.* 2006), and predicted data are available for four species, the dog, *Canis lupus familiaris* (GenBank accession no. XM_539386), the cat, *Felis catus* (Third Party

Figure 5. Schematic of the complement of single and double cone and rod photoreceptors in the avian retina as exemplified by many diurnal passerine species. Depending on the species, the SWS1 pigments have $\lambda_{\max}$ values either in the UV (360–380 nm) or in the violet (400–420 nm) region of the spectrum. Modified and redrawn from Bowmaker (2008).

Annotation Section of the DDBJ/EMBL/GenBank databases, accession no. TPA: BK006813), the polar bear and sea otter (Levenson *et al.* 2006). In all cases, Tyr86 is present, indicating that VS pigments are encoded in all four species.

The tuning of the VS pigments of New World and Old World primates (Bowmaker *et al.* 1991; Hunt *et al.* 1995) has been examined in detail by Yokoyama & Shi (2000), who found that the simultaneous replacement of the residues present at seven sites (Phe46Thr, Phe49Leu, Thr52Phe, Phe86Leu, Thr93Pro, Ala114Gly and Ser118Thr) in mouse UVS with those in human VS pigment produced a shift from 359 to 411 nm, and the reverse substitutions shifted the $\lambda_{\max}$ of the human VS pigment to 360 nm. Overall, therefore, these substitutions achieve the spectral shift between mouse UVS and human VS pigments, although their effects must be synergistic since single substitutions had no effect. Subsequently, it was shown (Shi *et al.* 2001) that although Leu86 and Pro93 substitutions into mouse UVS do not generate a spectral shift into the violet, the addition of substitutions at sites 114 and 118 results in a pigment with a $\lambda_{\max}$ at 399 nm. Since site 114 is not conserved across other primate species, the key substitutions in the generation of primate VS pigments from an ancestral UVS pigment would appear from this study to be Phe86Leu, Thr93Pro and Ser118Thr, although for the full shift to around 415 nm (Bowmaker *et al.* 1980), substitutions at sites 46, 49 and 52 may also be required (Shi *et al.* 2001).

Prosimian primates are divided into two strepsirrhine groups—the Lorisiformes and the Lemuriformes—and a haplorhine group—the Tarsiiformes (Fleagle 1999). Among the nocturnal lorisiforms, a functional *SWS1*

gene is absent (Jacobs *et al.* 1996b; Kawamura & Kubotera 2004), which contrasts with the lemuriforms and tarsiers in which functional *SWS1* genes are present (Kawamura & Kubotera 2004). Moreover, sensitivity to short wavelength light has been confirmed in the ringtail lemur (*L. catta*) and brown lemur (*Lemur macaco*), and the presence of S cones in the retina has been demonstrated in the grey mouse lemur and the eastern tarsier (*Tarsius spectrum*) (Hendrickson *et al.* 2000; Dkhissi-Benyahya *et al.* 2001). The *SWS1* genes in prosimians show considerable variability in the residues present at site 86, with Leu in tarsiers (as found in the pigments of Old and New World primates), Ser (grey mouse lemur, Coquerel's dwarf lemur, greater dwarf lemur and fat-tailed dwarf lemur), Cys (brown lemur and ring-tailed lemur), Val (woolly lemur), Leu (Verreaux's sifaka) and Phe (aye-aye) present. Cys, Ser, Leu and Val would all be expected to generate VS pigments, whereas Phe86 is generally associated with a UVS pigment (Hunt *et al.* 2004). Among the species with mutated pseudogenes, the lesser galago and slow loris have Tyr86, the greater and small eared galagos possess Asn86 and the pygmy slow loris has Cys86. By *in vitro* expression of the native *SWS1* gene sequences from the brown lemur (with Cys86), Coquerel's mouse lemur (with Ser86) and the aye-aye (with Phe86), plus a mutagenized brown lemur sequence encoding Val86 (as found in the woolly lemur), Carvalho *et al.* (2008) have shown that all specify VS pigments with $\lambda_{\max}$ ranging from 402 nm for the aye-aye pigment to 414 nm for the brown lemur pigment. Phe86 does not therefore result in a UVS pigment in the aye-aye, and the variation at site 86 would appear to fine tune the $\lambda_{\max}$ of the *SWS1* pigment in the different species. Consistent with this, the substitutions at this site have occurred at least four times in primate evolution (figure 4).

What change therefore was initially responsible for the red shift in the generation of the VS pigments seen in all primates? As shown in table 2, with the single exception of Pro93, the other sites that Shi *et al.* (2001) identified as responsible for this shift are not consistently substituted across all primate VS pigments. Only Pro93 is consistently present in these VS pigments and may represent therefore the initial change responsible for the generation of VS pigments at the base of the primate lineage.

(ii) LWS pigments

The molecular basis for the spectral tuning of mammalian LWS pigments was first established in primates. The LWS gene exists in two spectral forms in Old World primates that encode the L and M variants (Nathans *et al.* 1986; Ibbotson *et al.* 1992), with spectral peaks in humans and other Old World primates around 563 and 535 nm, respectively. Differences at only three sites are responsible for most of the spectral shift between pigments (Neitz *et al.* 1991); these are sites 164, 261 and 269, with the polar residues Ser, Tyr and Thr, respectively, in the L opsin and non-polar residues Ala, Phe and Ala in the M opsin (Asenjo *et al.* 1994). Minor shifts are associated with a Ser/Tyr substitution at site 100 and an Ile/Thr substitution at site 214. Site 164 is polymorphic in both the L and M genes in humans, with a consequent shift in the $\lambda_{\max}$ of the pigment.

New World primates use the same residue substitutions at sites 164, 261 and 269 to tune the different pigments encoded by the allelic variants of the LWS gene (Neitz *et al.* 1991; Williams *et al.* 1992). Site 261 does not vary in members of the family Callitrichidae so the L and M pigments differ by only 19–20 nm, compared with approximately 27 nm seen in Old World primates and in members of the other major New World family, the Cebidae, where site 261 is used. A feature of many New World primate species is the presence of three or more different LWS allelic variants that encode different combinations of substitutions at these three sites and generate spectrally different pigments (table 1). The L and M howler monkey pigments also show substitutions at these three sites (Jacobs *et al.* 1996a), consistent with the $\lambda_{\max}$ of the two pigments at approximately 530 and 558 nm (Saito *et al.* 2004).

A polymorphic LWS gene is also present in some prosimians (Tan & Li 1999). Different alleles were identified by DNA sequencing and classified as L or M on the basis of the amino acids encoded at the major tuning sites. Such variants were found in only three species: Coquerel's sifaka, the greater dwarf lemur and the red ruffed lemur, and for each species, two alleles were found that differed only at site 269, with Thr in the L pigment and Ala in the M pigment. The $\lambda_{\max}$ values for the two pigments were estimated by Tan & Li (1999) at 543 and 558 nm, and this has been confirmed by flicker photometry in one species, Coquerel's sifaka, where $\lambda_{\max}$ values around 545 and 558 nm were obtained (Jacobs *et al.* 2002). In a further 13 species in which it was possible to sample more than one X chromosome, only a single allele was found (Tan & Li 1999), although a second allele may

have been missed since the sample size for each species was small. Of these latter species, the majority have an M pigment (Ala269), with L pigments (Thr269) present in only three species. Trichromacy may have been present therefore in the common ancestor of the tarsiers and strepsirrhines, as revealed by the existence of L and M genes in present-day species, but only retained by a subset of these species.

Relatively few shifts in the $\lambda_{\max}$ of LWS pigments of non-primate species have been reported. The LWS pigment in the mouse is blue-shifted to 508 nm, as a result of the loss of the chloride-binding site. Many LWS pigments are anion-sensitive (Kleinschmidt & Harosi 1992), with His181 and Lys184 forming a chloride-binding site (Wang *et al.* 1993). Replacement of His181, which has occurred naturally in the mouse LWS pigment, abolishes chloride binding and generates a 28 nm short wavelength shift in $\lambda_{\max}$ (Sun *et al.* 1997). The $\lambda_{\max}$ of the LWS pigment of the bottlenose dolphin (*Tursiops truncatus*), an L cone monochromat, is also blue-shifted relative to terrestrial mammals, and a site-directed mutagenesis study (Fasick & Robinson 1998; Fasick *et al.* 1998) showed that the presence of Ser at site 292 was responsible. Among the mammals, Ser292 is only found in cetaceans (Newman & Robinson 2005), but is also present in the blue-shifted LWS pigment of the elephant shark, *Callorhynchus milii* (Davies *et al.* 2009). Using site-directed mutagenesis to generate a series of mutant pigments, Davies *et al.* (2009) have shown that Ser292 also acts via the inactivation of the chloride-binding site to blue-shift the $\lambda_{\max}$ of the pigment, even though His181 is still present.

3. PHOTORECEPTORS AND VISUAL PIGMENTS IN BIRDS

The arrangement and classes of photoreceptors within the retinae of birds are highly conserved across species with rod photoreceptors, double cones comprising a principal and accessory cell, and four spectral classes of single cone that provide for tetrachromacy. Birds have generally retained all four classes of cone visual pigments, with the LWS pigment expressed in members of both the double and single cones, and the other three cone pigment classes, Rh2, SWS2 and SWS1, each expressed in single cones (Hart & Hunt 2007). In diurnal birds, double cones comprise approximately 50 per cent of the total cone population, the LWS and RH2 single cones approximately 20 per cent each and the SWS2 and SWS1 single cones accounting for approximately 10 per cent (Bowmaker 2008). Birds appear to separate achromatic functions such as luminance, form and movement detection from chromatic tasks, using double cones for the former and single cones for the latter (reviewed in Osorio & Vorobyev 2005). Within the distal region of the inner segment, avian photoreceptors possess coloured oil droplets containing carotenoid pigments that are spectrally matched to cone pigments and act to cut off shorter wavelengths (figure 5). The principal member of double cones contains a large pale yellow, P-type droplet that cuts off at approximately 460 nm, whereas the accessory member

may contain either a small droplet or diffuse pigment throughout the inner segment. LWS cones contain a red R-type droplet that cuts off at approximately 560 nm, Rh2 cones have a yellow Y-type droplet with a cut-off at approximately 505 nm and SWS2 cones have a C-type droplet with a cut-off at 410–440 nm. SWS1 cones, which may be either VS or UVS, possess a transparent T-type droplet that shows no significant absorbance above 350 nm (Bowmaker *et al.* 1997; Hart *et al.* 2000).

An exception to the general pattern of LWS pigments in both members of double cones has been recently reported in the migratory bobolink (*Dolichonyx orizivorus*). This passerine species has double cones with a 565 nm pigment in the principal member, which presumably is encoded by the LWS gene, paired uniquely with a 403 nm pigment in the accessory member together with an oil droplet that cuts off below 410 nm (Beason & Loew 2008). LWS, MWS and UVS single cones are also present but no blue-sensitive or VS cones, implying that the SWS2 gene is not expressed in single cones. If expression of a very short wavelength-shifted SWS2 pigment was restricted to the accessory member of double cones, this could explain the unusual spectral characteristics of these cones. Recent molecular evidence, however, points to a duplicate copy of the SWS1 gene encoding the 403 nm pigment (Ödeen *et al.* 2009).

Relatively little is known about the cone complement in nocturnal bird species. In the few species that have been studied, a duplex retina is present but rod dominated with 80–90% rods compared with around 20–30% in diurnal species (Bowmaker & Martin 1978; Rojas *et al.* 2004). Cone spectral sensitivities have been studied in a single species, the tawny owl, *Strix aluco*, in which three classes have been found with $\lambda_{\max}$ values at 555, 503 and 463 nm, indicating the presence of the LWS, Rh2 and SWS2 pigments (Bowmaker & Martin 1978). Whether this is a complement of cones that is common to nocturnal species remains to be established, as does the presence of an SWS1 pigment.

(a) Spectral tuning of rod and cone visual pigments

(i) SWS1 pigments

In contrast to other vertebrates, avian UVS pigments were derived from ancestral avian VS pigments: UV sensitivity was therefore 're-invented' in birds. The key residue for an avian UVS pigment is the presence of Cys90 (Wilkie *et al.* 2000; Yokoyama *et al.* 2000b) rather than Phe86. Indeed, the residue site 86 in avian UVS pigments may be occupied by either Ala, Cys, Ile, Met or Phe (Wilkie *et al.* 2000; Yokoyama *et al.* 2000a,b; Ödeen & Håstad 2003), but never by Ser. This latter observation enabled Carvalho *et al.* (2007) to show that the substitution of Phe86 by Ser was the key change in the evolution of avian VS pigments.

Our understanding of the evolution of avian SWS1 pigments has been extended by a recent study of the gene sequence of these pigments in 46 bird species distributed across 14 avian orders

(Ödeen & Håstad 2003). The sequencing of a small region of the SWS1 gene that included sites 86–93 showed that although Ser90 is present in the SWS1 pigment of most species, a subset possesses Cys90 as follows: three of the 21 species from the order Ciconiiformes, four of the eight species from the order Passeriformes, both of the two species from the order Psittaciformes and one of the two species from the order Struthioniformes. From the phylogenetic relationships of the different orders (figure 6), it would appear that UVS pigments with Cys90 have evolved from the ancestral VS pigment at least four times. However, in many of the species examined by Ödeen & Håstad (2003), the $\lambda_{\max}$ values of the respective pigments have not been determined; therefore, it has yet to be fully established that all these species possess UVS pigments.

Phe86 is substituted in all but two of the avian SWS1 sequences listed by Ödeen & Håstad (2003). The two exceptions are the pigments of the common rhea, *Rhea americana*, and the blue-crowned trogon, *Trogon curucui*. In the former case, Cys90 is present, so that the pigment is most likely UVS, whereas Ser90 is present in the blue-crowned trogon, so either Phe86 has been retained in this species from the ancestral vertebrate UVS pigment, which would appear unlikely, or this represents a reverse mutation. Site-directed mutagenesis of the pigeon and chicken VS pigment confirms that the introduction of Phe86 shifts the $\lambda_{\max}$ into the UV (Carvalho *et al.* 2007), whereas contrary to the data of Shi & Yokoyama (2003), Cys86 does not.

(ii) SWS2 pigments

Only a few avian SWS2 pigments have been studied from a spectral and molecular perspective. $\lambda_{\max}$ values range from 440 nm in the zebra finch (Yokoyama *et al.* 2000a) and 442 nm in the canary (*Serinus canaria*) (Das *et al.* 1999) to 452 nm in the pigeon and 453 nm in the chicken (Okano *et al.* 1989; Bowmaker *et al.* 1997). None of the spectral tuning sites identified by Takahashi & Ebrey (2003) in their study of newt SWS2 pigments shows variation across the avian sequences, whereas site 269 does differ, with Ser or Thr present in the two or more red-shifted pigments (pigeon and chicken) compared with Cys in the two more blue-shifted pigments (canary and zebra finch). Thr/Ala substitutions at site 269 have been shown by site-directed mutagenesis (Cowing *et al.* 2002a) to be the underlying cause of an 11 nm shift in natural teleost SWS2 pigments, and a Ser269Cys substitution into the pigeon pigment was shown to act synergistically with a Val46Leu substitution to generate the spectral shift between the zebra finch and chicken/pigeon pigments (Yokoyama & Tada 2003). A complication is that the canary pigment with a similar $\lambda_{\max}$ to the zebra finch has retained Val46, so in this species, the Ser269Cys substitution would need to act synergistically with another substitution. Uniquely for avian SWS2 pigments, the canary pigment has Ser rather than Leu at site 207, another site identified by Yokoyama & Tada (2003) as important for the tuning of SWS2 pigments. This

Figure 6. Phylogenetic relationships showing the presence of VS and UVS SWS1 pigments in avian species so far examined. Blue lines are lineages with VS pigments and violet lines with UVS pigments. The ancestral avian VS pigment was most probably Ser86 and Ser90. Substitutions at these sites are shown on the respective branches. Re-drawn from Hunt *et al.* (2007).

may be therefore the additional substitution in the canary pigment that interacts with Cys269 to generate a blue shift.

(iii) *Rh1 and Rh2 pigments*

The rod *Rh1* gene arose from a duplication of the ancestral *Rh2* cone opsin gene (Okano *et al.* 1992) subsequent to the evolution of the four cone pigment classes in vertebrates. $\lambda_{\max}$ values for avian *Rh1* and *Rh2* pigments have been obtained for more than 20 species and in all cases, the two pigments show similar $\lambda_{\max}$ values with peaks between 497 and 509 nm. In general, *Rh1* and *Rh2* opsins differ at site 122, with Gln in *Rh2* replaced by Glu in *Rh1*. The replacement of Gln by Glu constitutes a non-conservative change (polar to charged residue), which when replicated by site-directed mutagenesis in bovine *Rh1* opsin results in a 15–20 nm shift to shorter wavelengths (Sakmar *et al.* 1989; Zhukovsky & Oprian 1989). If Gln122 in avian *Rh2* pigments causes a similar shift, then other substitutions must be present to compensate for this blue shift. All avian *Rh1* and *Rh2* pigments so far sequenced differ at site 222 with Cys in *Rh1* and Ser in *Rh2* and at site 295 with Ala in *Rh1* and Ser in

Rh2. As originally proposed by Heath *et al.* (1997), changes at these two sites may be responsible for the red shift in the $\lambda_{\max}$ of *Rh2* pigments to similar spectral locations as the *Rh1* pigments.

(iv) *LWS pigments*

The $\lambda_{\max}$ values of avian LWS pigments are mostly between 560 and 570 nm. Relatively few sequences are available, however, to assess the mechanisms of spectral tuning. As found in mammalian LWS pigments, a chloride-binding pocket determined by His181 and Lys184 (Wang *et al.* 1993) is present in avian LWS pigments (Okano *et al.* 1992; Das *et al.* 1999; Kawamura *et al.* 1999; Yokoyama *et al.* 2000a) and responsible for at least part of the long-wavelength shift.

LWS pigments are significantly shortwave-shifted in the Humboldt penguin *Spheniscus humboldti* at 543 nm (Bowmaker & Martin 1985) and the tawny owl *S. aluco* at 555 nm (Bowmaker & Martin 1978), but it has yet to be determined whether the blue shifts of these pigments are achieved by the replacement of polar residues at one or more common tuning sites identified for primate LWS pigments.

4. CONCLUSIONS

A striking feature of the avian visual system is how conserved it is across species, with all four cone opsin classes retained by most species and, except for the SWS1 classes, with peak sensitivities for the pigments showing little variation between species. The particular combination of morphological cone types, visual pigments and oil droplets would appear to be capable, therefore, of effective functioning across a wide range of light environments. A rod-dominant retina is however found in the few species of nocturnal birds that have been studied and birds are unique in initially losing UV sensitivity, although this has been re-acquired in many avian lineages by a single amino acid substitution. It should be noted however that the overall number of bird species that have been studied in depth remains small, so variation in cone complement and spectral tuning of pigments may yet turn out to be more common than presently indicated. In contrast, the study of visual pigments in a relatively large number of mammalian species has identified substantial variation. The basic cone complement has been reduced to only two classes—LWS and SWS2 in monotremes and LWS and SWS1 in eutherians and marsupials—and there is substantial variation in the peak sensitivities of the LWS pigment, together with gene duplications in primates to achieve trichromacy. UV sensitivity has been lost in many species, although unlike birds, it has been retained by some, and the loss of a functional *SWS1* gene pigment is common to most aquatic and many nocturnal species.

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