REVIEW

Spectral Tuning in Vertebrate Short Wavelength-Sensitive 1 (SWS1) Visual Pigments: Can Wavelength Sensitivity be Inferred From Sequence Data?

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FRANCES E. HAUSER[1], ILKE VAN HAZEL[1], AND BELINDA S. W. CHANG[1,2,3]*

[1]Department of Ecology & Evolutionary Biology, University of Toronto, Toronto, Ontario, Canada
[2]Department of Cell & Systems Biology, University of Toronto, Toronto, Ontario, Canada
[3]Centre for the Analysis of Genome Evolution & Function, University of Toronto, Toronto, Ontario, Canada

ABSTRACT

The molecular mechanisms underlying the enormous diversity of visual pigment wavelength sensitivities found in nature have been the focus of many molecular evolutionary studies, with particular attention to the short wavelength-sensitive 1 (SWS1) visual pigments that mediate vision in the ultraviolet to violet range of the electromagnetic spectrum. Over a decade of study has revealed that the remarkable extension of SWS1 absorption maxima (λ_{max}) into the ultraviolet occurs through a deprotonation of the Schiff base linkage of the retinal chromophore, a mechanism unique to this visual pigment type. In studies of visual ecology, there has been mounting interest in inferring visual sensitivity at short wavelengths, given the importance of UV signaling in courtship displays and other behaviors. Since experimentally determining spectral sensitivities can be both challenging and time-consuming, alternative strategies such as estimating λ_{max} based on amino acids at sites known to affect spectral tuning are becoming increasingly common. However, these estimates should be made with knowledge of the limitations inherent in these approaches. Here, we provide an overview of the current literature on SWS1 site-directed mutagenesis spectral tuning studies, and discuss methodological caveats specific to the SWS1-type pigments. We focus particular attention on contrasting avian and mammalian SWS1 spectral tuning mechanisms, which are the best studied among vertebrates. We find that avian SWS1 visual pigment spectral tuning mechanisms are fairly consistent, and therefore more predictable in terms of wavelength absorption maxima, whereas mammalian pigments are not well suited to predictions of λ_{max} from sequence data alone. *J. Exp. Zool. (Mol. Dev. Evol.) 9999B: XX–XX, 2014.* © 2014 Wiley Periodicals, Inc.

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Frances E. Hauser and Ilke van Hazel contributed equally to this study.
*Correspondence to: Belinda S. W. Chang, Department of Ecology & Evolutionary Biology, University of Toronto, 25 Harbord Street, Toronto, ON M5S 3G5, Canada.
E-mail: belinda.chang@utoronto.ca

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Studies of vertebrate vision are inherently interdisciplinary, utilizing a diverse array of experimental methods and conceptual frameworks, from studies of molecular evolution to behavioral assays. For example, behavioral studies often incorporate models that describe how color signals are detected and perceived by an observer (Vorobyev et al., '98; Endler et al., 2005). The parameters of these models are ideally based on visual pigment spectral absorbance data from the species of interest, but the availability of such information can be limited. For instance, because experimentally determined spectral absorbance data exists for only a small proportion of bird species (Hart, 2001; Hart and Vorobyev, 2005; Coyle et al., 2012), researchers may model the visual abilities of birds through estimations derived from related species. However, any inferences of visual pigment spectral sensitivity should be implemented with full knowledge of the limitations associated with such estimations. This is particularly the case for short wavelength-sensitive opsins. In contrast to other vertebrate cone visual pigments, SWS1 pigments have an extremely broad variation in wavelength of maximal absorbance (λ_{max}), spanning a range of almost 100 nm. Moreover, large shifts in λ_{max} spectral sensitivities (>30 nm) occur across the vertebrate phylogeny, even in closely related linages (Carvalho et al., 2012). As an alternative to inferring the visual pigment λ_{max} of a species of interest using data from related species, λ_{max} may be estimated based on the amino acid residues present at known spectral tuning sites identified in site-directed mutagenesis experiments (Yokoyama, '95; Ödeen and Håstad, 2003; Ödeen et al., 2009). This approach has been implemented in numerous recent studies (e.g., Håstad et al., 2005; Ödeen and Håstad, 2009, 2010; Ödeen et al., 2010, 2011, 2012; Machovsky Capuska et al., 2011; Aidala et al., 2012).

The accuracy of λ_{max} estimates based on sequence data alone has not been fully explored in SWS1 pigments, despite their increasing importance for studies of visual ecology and behavior. In this paper, we review visual pigment spectral tuning mechanisms, and highlight key differences between SWS1 pigments and other visual pigments. We summarize what is known of SWS1 pigment wavelength regulation from mutagenesis and protein expression studies in vertebrates, with particular attention to the birds and mammals. This review examines the feasibility of inferring λ_{max} based on sequence data in these two groups, and highlights issues with respect to experimental determination of λ_{max}, as well as the potential challenges these present for behavioral inferences of visual performance.

SPECTRAL TUNING IN VISUAL PIGMENTS

SWS1 pigments exhibit broad natural variation in λ_{max}, ranging from violet to UV. Accordingly, vertebrate SWS1 pigments have been divided into two sub-types on the basis of spectral sensitivity (Fig. 1; Hunt et al., 2009; Davies et al., 2012): UV sensitive (UVS: λ_{max} 355–380 nm) and violet sensitive (VS: λ_{max} 388–455 nm). Though both subtypes have been identified in most vertebrate groups, their distribution across the vertebrate tree is highly variable, and phylogenetic studies have demonstrated that spectral shifts between the two subtypes have likely occurred several times throughout vertebrate evolution (Yokoyama and Shi, 2000; Shi et al., 2001; Cowing et al., 2002; Takahashi and Ebrey, 2003; Parry et al., 2004; Carvalho et al., 2007, 2012; Ödeen and Håstad, 2013). These studies suggest the vertebrate ancestral state was UVS, with VS pigments subsequently evolving in various lineages independently (reviewed in Yokoyama and Shi, 2000; Hunt et al., 2007). Birds, on the other hand, likely possessed an ancestral VS pigment with certain lineages regaining UVS (Shi et al., 2001).

Visual pigment spectral tuning is a common mechanism by which vertebrates are thought to adapt their vision to diverse light environments (Davies et al., 2012). As the first step in the visual transduction cascade that converts light into a neural signal (Wald, '68), visual pigments constitute a crucial initial component of sensory visual function in vertebrates. Structurally, a visual pigment consists of an opsin protein covalently bound to a light sensitive chromophore (11-*cis*-retinal) via a protonated Schiff base linkage. Opsin proteins are members of the G protein-coupled receptor superfamily, and possess a characteristic seven transmembrane helical structure forming a binding pocket around the retinal chromophore (Smith, 2010) (Fig. 2). Absorption of a photon of light by the retinal chromophore induces a *cis* to *trans* isomerization that subsequently causes a conformational change

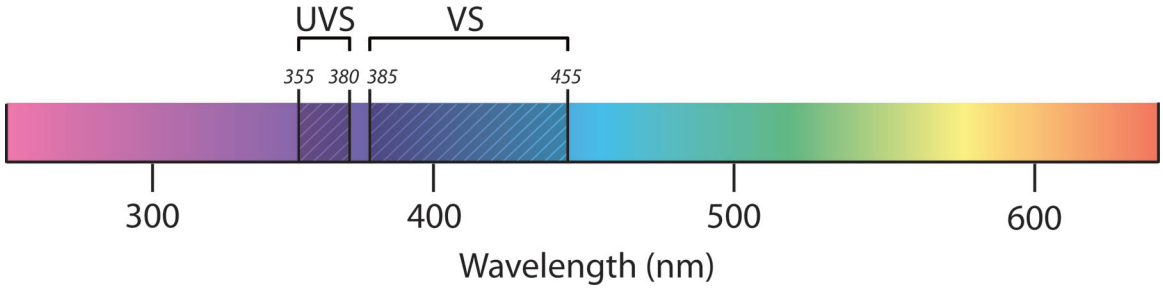

Figure 1. Spectral range of absorbance of vertebrate short-wavelength sensitive 1 (SWS1) pigments.

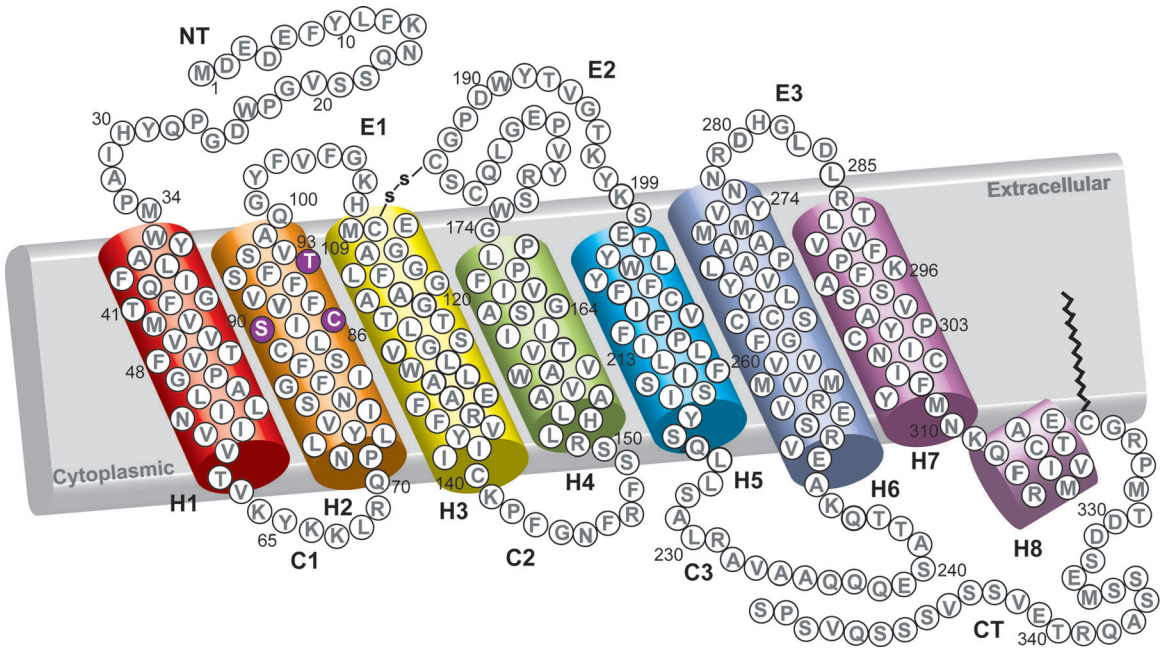

Figure 2. Location of SWS1 spectral tuning sites. The bowerbird SWS1 sequence is displayed, with protein domains based on the bovine RH1 crystal structure (Palczewski et al., 2000). The seven transmembrane α-helices (H1–H7), Helix 8 (H8), extracellular (E1–E3) and cytoplasmic (C1–C3) regions, as well the cytoplasmic terminal region (CT) and the N-terminal region (NT) are labeled. Amino acid positions refer to bovine RH1 numbering. Major spectral tuning sites 86, 90, and 93 are shaded in purple.

in the protein, leading to the activation of the G protein transducin by the activated form of the opsin protein, metarhodopsin II (MII) (Pugh and Lamb, '93). G protein activation initiates a biochemical cascade within the photoreceptor that ultimately results in a neural signal that light has been detected (Menon et al., 2001). In addition to SWS1, there are three other cone pigment types in vertebrates: long-, middle-, and a second short-wavelength-sensitive pigment (LWS, RH2, SWS2) (Bowmaker, 2008). These pigments mediate bright-light (photopic), and (in some cases) color vision through the integration of signals from multiple cone photoreceptors (Baccus, 2007; Mustafi et al., 2009). The fifth group of visual pigments comprises the rod opsins (also known as rhodopsins or RH1). Unlike cone pigments, rod opsins mediate vision in dim light (scotopic vision), and the spectral range of λ_{max} tends to be more restricted. The variations in spectral sensitivity within and among the other visual pigment types will not be discussed in detail here, but have been thoroughly reviewed elsewhere (e.g., Davies et al., 2012).

Although the protonated Schiff base form of the retinal chromophore is known to absorb at 440 nm in solution, the vertebrate opsin protein environment interacts with the chromophore such that the absorption maximum of retinal can be altered over a wide spectral range, a phenomenon referred to as the opsin shift (Honig et al., '79; Kleinschmidt and Harosi, '92). Spectral tuning of visual pigments is accomplished through interactions between amino acid side chains within the binding pocket of the opsin protein and its associated chromophore (Kochendoerfer et al., '99). These amino acid residues lining the binding pocket influence the ground and excited transition energies of the chromophore, affecting the energy required for activation (Lin et al., '98). In visual pigments, amino acid substitutions are thought to influence this opsin shift via several mechanisms: (1) modifying the strength of the electrostatic interaction between the protonated Schiff base and its counterion (Blatz et al., '72; Baasov et al., '87); (2) changing the position of charges along the polyene chain (Honig et al., '79); and (3) twisting of the polyene chain due to the protein environment (Blatz and Liebman, '73; Kakitani et al., '85) (reviewed in Kochendoerfer et al., '99). Nevertheless, amino acid substitutions are not the sole mechanism by which visual pigment spectral sensitivity can be altered. In some vertebrate groups, spectral tuning is also achieved through the replacement of the A1 chromophore (11-*cis*-retinal) with A2 (11-*cis*-3,4-dehydroretinal), which red-shifts λ_{max} up to 20 nm (Harosi, '94; Yokoyama, 2000). At the photoreceptor level, pigmented cone oil droplets can also act as cut-off filters, impeding transmission of certain wavelengths of light into the visual pigment-containing region of photoreceptor outer segments (Bowmaker and Knowles, '77; Bowmaker et al., '97).

SWS1 visual pigments are the only opsin type that can have λ_{max} values shifted from the 440 nm absorption maximum of the protonated Schiff base retinal chromophore into the UV range (λ_{max} values < 380 nm). Because the SWS1 cone oil droplet generally does not filter short-wavelength light (Bowmaker, '80; Hart, 2004; Coyle et al., 2012), and usage of A1 vs. A2 chromophore has a negligible effect on λ_{max} in this range (Kawamura and Yokoyama, '98), amino acid replacements are thought to be primarily responsible for this breadth in spectral sensitivity. The absorbance shift into the UV is achieved through deprotonation of the Schiff base linkage in the dark-state (Kusnetzow et al., 2004; Hunt et al., 2007; Altun et al., 2009), a characteristic unique to SWS1 visual pigments. All other visual pigments contain protonated Schiff base chromophore linkages, with the negatively charged E113 serving as the counterion to stabilize this proton (Nathans, '90). In rod pigments, residues found at sites 94, 113, 181, 186, 192, and 268 are involved in a hydrogen bond network that also aids in stabilization of the protonated Schiff base (Janz and Farrens, 2004). In SWS1 pigments, four of these residues are identical to those found in rods, and there are no differences at these sites between UV-sensitive (UVS) and violet-sensitive (VS) SWS1. The structural factors that determine this distinct protonation state of the chromophore in UVS pigments have not yet been fully elucidated at the molecular level, though a recent study has implicated water molecules participating in the E113 bond network in a mammalian UV pigment (Mooney et al., 2012). The absence of Schiff base protonation in UVS SWS1 is particularly intriguing because it eliminates one of the dark-state activation barriers that is present in all other visual pigment classes: ionic interactions between the protonated Schiff base linkage and the counterion are thought to suppress activation of the second messenger G protein transducin. In bovine rhodopsin, counterion mutations such as E113Q disrupt this interaction, and result in constitutively active receptors (Tsutsui et al., 2007; reviewed in Tsutsui and Shichida, 2010). How constitutive activity is suppressed in UV pigments with an unprotonated Schiff base linkage is unclear, but there is evidence that E113 is still involved, as this residue is conserved even in UV pigments (Kono, 2009). Subsequent protonation of the light-activated photointermediate occurs prior to the formation of the transducin-activating metarhodopsin II state in all visual pigments, including UVS SWS1 (Kusnetzow et al., 2004; Mooney et al., 2012). Though we focus on spectral tuning mechanisms in this review, UVS and VS SWS1 pigments also differ in other physicochemical characteristics aside from absorption maxima, and these differences are likely influenced by the protonation state of the Schiff base. It should be noted, however, that an aromatic residue near the β-ionone ring of the chromophore in SWS1 pigments has also been shown to mediate non-spectral properties in these pigments (Kuemmel et al., 2013). UVS SWS1 pigments have been found to possess a slower retinal release (Chen et al., 2012), a more tightly packed binding pocket (Das et al., 2004), increased dark state stability (Luo et al., 2011), and a narrower absorption curve bandwidth relative to VS pigments (Govardovskii et al., 2000; Tsutsui and Shichida, 2010).

Spectral tuning in visual pigments has been investigated using a variety of approaches, including comparative sequencing, microspectrophotometry, site-directed mutagenesis, and *in vitro* expression (see Hunt et al., 2004; Bowmaker and Hunt, 2006; Yokoyama, 2008; Hunt et al., 2009; Davies et al., 2012, and references therein). Numerous spectral tuning sites have been proposed for all visual pigment groups, but for the purpose of this review we will focus on spectral tuning sites in SWS1 visual pigments as identified by site-directed mutagenesis experiments, in which a direct link between amino acid substitutions and shifts in wavelength sensitivity has been conclusively demonstrated.

SITE-DIRECTED MUTAGENESIS STUDIES OF SWS1 SPECTRAL TUNING

The impressive structural and functional variation found among SWS1 opsins has motivated a number of mutagenesis studies to determine the underlying mechanisms (e.g., Takahashi and Yokoyama, 2005; Hunt et al., 2009; Carvalho et al., 2010, 2012). One of the most striking results to emerge from these studies is that substitutions at individual amino acid sites are largely responsible for the dramatic shifts in λ_{max} between UVS and VS SWS1 pigments. Moreover, across vertebrates, these point mutations of large effect can vary substantially in amino acid identity and location (Wilkie et al., 2000; Yokoyama et al., 2000, 2005; Fasick et al., 2002; Cowing et al., 2002; Parry et al., 2004). However, in other cases, particularly in primates, a large λ_{max} shift is accomplished through the collective effect of several residues (Fasick et al., '99; Yokoyama and Shi, 2000; Takahashi and Yokoyama, 2005; Yokoyama et al., 2006; Carvalho et al., 2012).

Generally speaking, the most important residues for SWS1 spectral tuning in vertebrates have been found to be 86, 90, and 93 (Table 1; all numbering according to bovine rhodopsin), with additional sites mediating less pronounced effects. Although substitutions at sites 86, 90, and 93 can cause dramatic shifts between UV/violet, these effects are not consistent across all vertebrates. Here, we provide an overview of SWS1 spectral tuning mutations in different vertebrate groups that have been elucidated through *in vitro* expression and site-directed mutagenesis studies, with a view toward determining vertebrate groups in which the effects of amino acid substitutions appear most consistent, and therefore useful for λ_{max} estimation.

Fishes, Amphibians, and Reptiles

All fish studied to date possess a UVS SWS1 pigment, with many fish losing the SWS1 cone as they mature (Hunt et al., 2007). The only VS SWS1 in fish currently reported is found in the scabbardfish, where a deletion of F86 appears to have resulted in a λ_{max} of 423 nm (Tada et al., 2009). Site-directed mutagenesis of goldfish UVS SWS1 has shown that F86M and F86L mutants produced pigments nearly identical to wild-type, but the

Table 1. SWS1 *in vitro* expression and site-directed mutagenesis in vertebrates.

Species	Mutation	Measured λmax (nm)	Shift from WT (nm)	Refs.
Ancestral vertebrate	F49V/F86S/L116V/S118A	393	+33	Shi and Yokoyama (2003)
Scabbardfish	WT has deletion of F86	423	WT	Tada et al. (2009)
Goldfish	WT	358	WT	Cowing et al. (2002)
Goldfish	F86Y	420	+62	Cowing et al. (2002)
Goldfish	F86V	359	+1	Cowing et al. (2002)
Goldfish	F86S	363	+5	Cowing et al. (2002)
Goldfish	F86L	358	0	Cowing et al. (2002)
Goldfish	F86Y	413	+55	Hunt et al. (2004)
Goldfish	F86S	363	+5	Hunt et al. (2004)
African clawed frog	WT	427	WT	Starace and Knox ('98)
Python	WT	360	WT	Davies et al. (2009)
Green anole	F86S	370	+10	Carvalho et al. (2007)
Ancestral avian	S86C	366	−27	Shi and Yokoyama (2003)
Ancestral avian	S90C	360	−33	Shi and Yokoyama (2003)
Ancestral avian	S86C/S90C	360	−33	Shi and Yokoyama (2003)
Ancestral avian	F46L/S86C/S90C/A114G	360	−33	Shi and Yokoyama (2003)
Cormorant	WT	405	WT	Carvalho et al. (2007)
Budgerigar	WT	360	WT	Carvalho et al. (2007)
Budgerigar	C90S	420	+60	Hunt et al. (2004)
Budgerigar	C90S	398	+35	Wilkie et al. (2000)
Budgerigar	A86S	362	−1	Wilkie et al. (2000)
Budgerigar	A86S	361	+1	Carvalho et al. (2007)
Budgerigar	A86S/C90S	–	nonfunctional	Carvalho et al. (2007)
Zebra finch	WT	359	WT	Yokoyama et al. (2000)
Zebra finch	C90S	397	+38	Yokoyama et al. (2000)
Pigeon	WT	393	WT	Yokoyama et al. (2000)
Pigeon	S90C	359	−34	Yokoyama et al. (2000)
Pigeon	WT	388	WT	Carvalho et al. (2007)
Pigeon	S86A	384	−4	Carvalho et al. (2007)
Pigeon	S90C	359	−29	Carvalho et al. (2007)
Pigeon	S86F	357	−31	Carvalho et al. (2007)
Pigeon	S86C	386	−2	Carvalho et al. (2007)
Bowerbird	WT	403	WT	van Hazel et al. (2013)
Bowerbird	C86F	370	−33	van Hazel et al. (2013)
Bowerbird	C86S	403	WT	van Hazel et al. (2013)
Chicken	WT	419	WT	Carvalho et al. (2007)
Chicken	S90C	369	−46	Yokoyama et al. (2000)
Chicken	S86F	372	−47	Carvalho et al. (2007)
Cow	WT	435	WT	Cowing et al. (2002)
Cow	Y86F	363	−72	Cowing et al. (2002)
Cow	Y86S	422	−13	Cowing et al. (2002)
Cow	S90C	431	−4	Fasick et al. (2002)
Elephant	S86F	367	−52	Yokoyama et al. (2005)
Guinea pig	V86F	367	−53	Parry et al. (2004)
Mouse	F46T/F49L/T52F/L81F/ F86L/T93P/A114G/S118T	412	+53	Shi et al. (2001)
Mouse	F86Y	424	+66	Fasick et al. (2002)
Coquerel's mouse lemur	F86S	416	+7 nm	Carvalho et al. (2012)
Brown Lemur	C86V	401	−12 nm	Carvalho et al. (2012)
Aye-aye	P93T	371	−35 nm	Carvalho et al. (2012)
Human	S90C	417	−7	Fasick et al. ('99)

substitution F86Y red-shifted the UVS pigment by 60 nm, producing a VS pigment (Cowing et al., 2002). Although most amphibians possess UVS SWS1, opsin expression studies have demonstrated that frogs are VS, with M86 and P93 residues likely responsible for this shift (Starace and Knox, '98; Takahashi and Yokoyama, 2005). Because F86M did not produce a pronounced shift in goldfish SWS1, site 93 may instead contribute to a more substantial red shift in these pigments, as we discuss in mammals. Thus far, no instances of VS SWS1 pigments have been experimentally recorded in reptiles, and they are inferred to all possess UVS pigments due to the presence of F86 (Kawamura and Yokoyama, '96; Yokoyama and Blow, 2001). However, functional characterization of SWS1 pigments across reptilian taxa has not been performed extensively (but see Davies et al., 2009).

Mammals

In mammal SWS1 pigments, site 86 is known to be important for spectral tuning. Substitutions at this site can result in large λ_{max} shifts into the UV; for example, Y86F in bovine SWS1 (−71 nm; Cowing et al., 2002; Fasick et al., 2002), V86F in guinea pig (−53 nm; Parry et al., 2004), and S86F in the elephant pigment (−52 nm; Yokoyama et al., 2005). Substitutions replacing F86 shift λ_{max} into the violet in the UVS mouse pigment (F86Y, +66 nm; Fasick et al., 2002). However, site 90, which is important in birds, does not seem to play a role in spectral tuning in mammalian SWS1 pigments, where it is conserved as serine (Hunt et al., 2004).

Although there is mounting evidence that site 86 is an important spectral tuning site in many mammalian lineages, it is certainly not the sole spectral tuning substitution in mammals, and its role is not shared across the entire class. First, in some primate lineages a collection of sites confer the loss of UVS, as opposed to a single site. For instance, there are eight primary sites responsible for the λ_{max} difference between the human VS pigment and the mouse UVS pigment (Yokoyama and Shi, 2000; Shi et al., 2001). In this case, F46T, F49L, T52F, L81F, F86L, T93P, A114G, and S118T replacements in mouse UVS SWS1 do not cause a shift in λ_{max} individually, but together provide a strong synergistic effect, shifting λ_{max} +53 nm (Shi et al., 2001). The reverse substitutions shifted the human VS SWS1 −55 to 359 nm (Shi et al., 2001). However, across primates only sites 49, 86, 93, and 118 are conserved, so a definitive characterization of SWS1 spectral tuning in this group remains elusive. Second, the ability of F86 to alter sensitivity is not always consistent. This is evident in the aye-aye (a Madagascan lemur), which has a VS SWS1 despite also having F86 (Carvalho et al., 2012). This effect may be due to variation in electrochemical properties of nearby residues preventing the deprotonation of the Schiff base by F86. Instead, P93T significantly shifts λ_{max} (−35 nm into the UV) in the aye-aye. In these primates, a shift to VS from a UVS ancestor is likely mediated through a substitution at site 93, regardless of the residue found at site 86 (Carvalho et al., 2012). This observation implies that although primates may share the VS state, the human SWS1 pigment has evolved to be markedly different from other VS-type pigments in primates. The variability in the effect of sites 93 and 86 in regulating λ_{max} also demonstrates that critical changes can occur over short evolutionary distances in mammalian lineages.

Birds

In contrast to mammals, spectral tuning in bird SWS1 pigments is more straightforward; only a few sites have been identified that show similar effects on λ_{max} in all avian SWS1 pigments examined to date. In bird SWS1 pigments, site 90 has been found to be the most important residue in determining spectral sensitivity (Wilkie et al., 2000; Yokoyama and Shi, 2000; Yokoyama et al., 2000; Shi and Yokoyama, 2003), with the substitution S90C in avian VS pigments consistently found to shift λ_{max} into the UV (Wilkie et al., 2000; Yokoyama et al., 2000). Site 86 has also been found to have some effect in birds; shifts from VS to UVS in SWS1 are found with substitutions toward F86 (Carvalho et al., 2007; van Hazel et al., 2013). Curiously, paleognaths (ratites and tinamous) possess F86, but physiological and MSP data instead support the presence of violet type SWS1 (Wright and Bowmaker, 2001; but see Hunt and Peichl, 2013). Other substitutions at site 86 have been found to have variable effects. A hypothetical ancestral avian SWS1 demonstrated that a 30 nm shift into the UV could be mediated by S86C (Shi and Yokoyama, 2003), but in pigeon SWS1 this same substitution S86C does not produce a shift in λ_{max} (Carvalho et al., 2007). The reverse substitution (C86S) had no effect on λ_{max} in the bowerbird VS pigment (van Hazel et al., 2013). Although site 93 does show some variation among bird SWS1 pigments, in mutagenesis studies it tends to have a negligible effect on λ_{max}, and does not appear to modulate the effects of F86 or C90 (Wilkie et al., 2000; Yokoyama et al., 2000; Shi and Yokoyama, 2003; Carvalho et al., 2007; van Hazel et al., 2013).

An aspect of SWS1 spectral tuning that has received comparatively little attention concerns the variation in maximal absorption of VS SWS1 pigments. The few avian VS pigments that have been studied experimentally, the pigeon and chicken, differ considerably in their λ_{max} values (388 and 429 nm, respectively; Fager and Fager, '81; Carvalho et al., 2007). How VS pigments with such different wavelength sensitivities might influence visual performance is currently unknown. Given that the spectral difference between these pigments (∼30 nm) is as broad as some UVS vs. VS SWS1 pigment differences (e.g., pigeon VS vs. zebra finch UVS, ∼28 nm), it seems plausible that the disparity among VS-type SWS1 pigments may impart differences in color perception that are as important as a UVS/VS difference, since even very small differences in λ_{max} can be important in other vertebrates (e.g., Seehausen et al., 2008). Previous work has suggested that S86C may contribute to variation in VS pigments because it shifted λ_{max} in the hypothetical ancestral avian SWS1

(Shi and Yokoyama, 2003). As we have described, S86C does not appear to alter λ_{max} in a number of wild type pigments, so the mechanism regulating the difference in λ_{max} among these VS pigments remains unknown. Sites 49, 93, 118, and 298 are potential candidates, as they were able to modulate λ_{max} in ancestral vertebrate and extant mammalian pigments. Substitutions at these sites may not alter sensitivity in UVS pigments, likely due to the strong UV-shifting effect of C90, but their role in VS pigments has not been investigated in detail. Furthermore, in chicken SWS1 sites 93, 118, and 298 show interesting amino acid variation relative to other avian VS pigments. In basal primates, site 93 can affect sensitivity on its own (Carvalho et al., 2012), but can also regulate the effects of other sites, such as 118 (Shi et al., 2001), and therefore may serve a similar function in birds.

Can λ_{max} Be Inferred From Sequence Data?

Mutagenesis studies present a strong case that, depending on the visual pigment, sites 90 and 86 can have disproportionately large effects on SWS1 λ_{max}. Although we can broadly speculate on whether a pigment is UVS or VS based on these residues, can we assume that C90 and F86 will cause a UV shift in all circumstances? In mammals, it seems that predicting λ_{max} based on amino acid residues alone could be difficult, because the role of individual spectral tuning sites is less consistent even among closely related lineages. Within mammals, as well as across the major vertebrate groups, there is considerable variation in SWS1 spectral tuning sites, and the magnitude of λ_{max} shift caused by a given amino acid substitution can differ significantly (Shi et al., 2001; Takahashi and Ebrey, 2003; Hunt et al., 2004; Parry et al., 2004; Takahashi and Yokoyama, 2005; Hunt et al., 2009; Carvalho et al., 2012). Mammalian studies also highlight the importance of other residues in indirectly modulating the effects of spectral tuning residues near the protonated Schiff base (Shi et al., 2001; Fasick et al., 2002; Takahashi and Yokoyama, 2005; Carvalho et al., 2012). For example, in human and bovine SWS1 pigments substituting Cys at site 90 does not shift λ_{max} into the UV (Fasick et al., '99; Fasick et al., 2002), suggesting that important changes occurred during the evolution of avian SWS1 pigments that facilitated the evolution of C90 as a UV shifting residue.

Avian SWS1 pigments provide a more reliable framework for inference of λ_{max} based on the identities of amino acids at sites known to affect spectral tuning. Both S90C and S86F have been found to have consistent effects in a variety of bird pigments, shifting λ_{max} into the UV (Wilkie et al., 2000; Yokoyama et al., 2000; Hunt et al., 2004; Carvalho et al., 2007; van Hazel et al., 2013). However, while it may be feasible to make general inferences of UV vs. VS in bird SWS1 pigments based on sequence alone, precise estimation of λ_{max} values is unlikely to be reliable until further mutagenesis experiments are conducted, particularly with respect to the range found within avian VS pigments. It is important to remember that current mutagenesis studies are limited to the pigeon (Hunt et al., 2004; Carvalho et al., 2007), chicken (Carvalho et al., 2007), bowerbird (van Hazel et al., 2013), budgerigar (Wilkie et al., 2000), and zebra finch (Yokoyama et al., 2000), and that these studies mainly emphasize residues responsible for spectral differences between the VS and UVS pigments, rather than residues governing within-type variation. Sequence surveys focusing on the region containing known spectral tuning sites of the SWS1 gene have identified considerable variation, even at sites 86 and 90 (Ödeen and Håstad, 2003, 2009; Håstad et al., 2005; Ödeen et al., 2009, 2011). The implications of this variation for spectral tuning have yet to be explored in mutagenesis experiments. Thus, it is possible that in these varied background conditions the effects of F86 and C90 may differ. Overall, because other spectral tuning sites may exist, any prediction that F86 and C90 will UV shift λ_{max} across the entire avian lineage should be made with these caveats in mind.

EXPERIMENTAL CHALLENGES IN DETERMINING SWS1 λ_{MAX}

In all visual pigments, different curve-fitting methods used to estimate λ_{max} values, whether by fitting an opsin template to a dark spectrum or a difference spectrum, can give different λ_{max} estimates (Fasick et al., 2002). Curve fitting can prove particularly challenging in SWS1 pigments due to Rayleigh scattering of short wavelength light, which can distort the measured absorbance spectra in the UV region (Wilkie et al., 2000). Additionally, λ_{max} estimates measured *in vivo* by microspectrophotometery (MSP) may differ from those made *in vitro* by expression of pigments. For instance, the λ_{max} of chicken SWS1 estimated from *in vitro* expressed pigments can vary by several nm (415 nm, Yokoyama et al., 2000; 419 nm, Carvalho et al., 2007), while the λ_{max} inferred from the purified eye pigment is 417 nm (Fager and Fager, '81), and from MSP of intact photoreceptors is 418 nm (Bowmaker et al., '97).

Estimates of λ_{max} inferred from *in vitro* expression experiments can be affected by several factors that alter the shape of the absorption spectrum such that it does not conform to the standard opsin template curve. First, peak absorbance may be affected by the underlying absorbance of buffers in the UV range. Second, the presence of secondary absorbance peaks can be problematic, particularly for curve fitting estimation of λ_{max} (Govardovskii et al., 2000), which do not take these factors into account. However, estimating λ_{max} from difference spectra might correct for this (Fasick et al., '99; Wilkie et al., 2000). Third, lower yields due to decreased stability may also interfere with accurate λ_{max} estimation. This is particularly important because lower yields tend to produce noisier data, making accurate estimation of λ_{max} values more difficult.

Taking these issues into account, it is possible that some of the observed variation in λ_{max} among vertebrate SWS1 pigments might be an artifact of the variety of methods used to determine λ_{max} and their inherent issues. Comparing differences in estimation methods for various types of spectral data, and determining the degree to which poor data affects λ_{max} estimates

may be beneficial. Chicken SWS1 would serve as an appropriate model since it has been measured multiple times using a variety of methods.

Secondary Absorbance Peaks

SWS1 pigments present unique issues in estimating λ_{max} values. When expressed in solution, SWS1 can often have abnormal absorption spectra that have been described as "broad" or as having an "additional minor peak", whose cause remains currently unknown (Fasick et al., '99; Wilkie et al., 2000; Yokoyama and Shi, 2000; Yokoyama et al., 2000; Babu et al., 2001; Dukkipati et al., 2001; Shi et al., 2001; Tsutsui and Shichida, 2010). In VS pigments, this minor peak occurs at 410–420 nm, so the culprits are likely not active photointermediates metarhodopsin II ($\lambda_{max} \sim 380$ nm), or III ($\lambda_{max} \sim 470$ nm). Excess retinal may form adducts with phospholipid head-groups that absorb at λ_{max} 440–450 nm (Sommer and Farrens, 2006). Among the UV absorbing pigments, the additional peak occurs at 380 nm, so in this case metarhodopsin II is a possibility. Nevertheless, it is also possible that this additional peak is caused by unprotonated and protonated Schiff base forms of the pigment existing in equilibrium (Babu et al., 2001). Thus far, attempts to narrow the absorption spectrum experimentally (for example, by altering pH conditions and increasing yield) have been unsuccessful (Yokoyama et al., 2000; Shi et al., 2001; Tsutsui and Shichida, 2010; van Hazel et al., 2013).

Why the absorption spectra of SWS1 pigments can be broader than others remains unclear. To our knowledge, broad bandwidth spectra have never been observed in microspectrophotometry (MSP) data, where absorbance spectra of visual pigments are measured in the outer segments of photoreceptor cells. The fact that UVS type pigments have characteristically narrow absorbance spectra *in vivo* (e.g., Govardovskii et al., 2000) suggests that the additional peak is likely due to some structural change in the pigment when it is not in its native membrane environment. In RH1 pigments, the detergent used can affect the transition through each intermediate (Lewis et al., '97; Heck et al., 2003; Kuwayama et al., 2005), which may be responsible for the secondary peak if a photointermediate is formed. For this reason, it would be useful to study these pigments in different detergents to determine if the formation of secondary peaks that broaden the spectra can be avoided.

CAVEATS AND LIMITATIONS

Using spectral tuning residues to estimate λ_{max} is a cost and time efficient method, which can produce testable functional hypotheses. Estimating λ_{max} based on residues at particular spectral tuning sites is being increasingly incorporated into studies of visual behavior and ecology, and may be applicable in certain cases, such as with avian SWS1 pigments. However, these inferences concerning wavelength sensitivity must be approached with care, and may not be appropriate to all vertebrate groups. While mutagenesis work in avian SWS1 spectral tuning has consistently identified F86 and C90 as important substitutions, studies in mammals have revealed a diversity of spectral tuning sites (Hunt et al., 2004, 2009; Carvalho et al., 2012 and references therein). Furthermore, partitioning SWS1 λ_{max} as either UVS or VS does not account for potentially dramatic wavelength differences among VS type pigments, which can vary from 390 to 419 nm in birds (Yokoyama et al., 2000; Carvalho et al., 2007), and from 406 to 460 nm in mammals (Hunt et al., 2009; Hunt and Peichl, 2014).

The unique experimental issues encountered in expressed SWS1 pigments present additional challenges in estimating λ_{max} from sequence data. For instance, current calculations implemented in birds are based on early mutagenesis studies where C90S shifts the λ_{ma} of budgerigar UVS pigment (wild-type $\lambda_{max} = 363$ nm) by 35 nm (Wilkie et al., 2000). Subsequent experiments with improved protein yield revealed a shift closer to 60 nm from wild type λ_{max} (Hunt et al., 2004). Although C90 and F86 appear to have large, consistent effects on λ_{max} in all avian SWS1 pigments, a lack of understanding of spectral tuning mechanisms among VS type pigments precludes reliable estimates of precise λ_{max} values in this pigment type until further expression work is conducted.

It should also be noted that while this review focuses on the use of site-directed mutagenesis and *in vitro* expression to study the effects of single amino acid substitutions on visual pigment function, there are of course limitations associated with these approaches. As with all *in vitro* approaches, it can be difficult to extrapolate what the effects of substitutions might be in an organismal context. It is possible that interactions with other components of the visual transduction cascade within the photoreceptor cell might be affected, or even other aspects of visual physiology that would be difficult to predict from *in vitro* studies. Although few mutations in SWS1 pigments have been studied in transgenic animals (e.g., Insinna et al., 2012), this is clearly an interesting area of future study.

Use of λ_{max} Estimation in Behavioural Studies

The elucidation of λ_{max} through gene sequence analysis, protein expression, and MSP plays a vital role in our exploration of visual system function and evolution. However, behavioral manifestations of these genetic, physiological, and neural mechanisms are also important to our understanding of visual system performance. The limitations of λ_{max} estimation we have outlined above should be carefully considered when attempting behavioral assays, particularly in the design of stimuli. Examples of discrepancy between inferred and measured λ_{max} can be found throughout the SWS1 literature, particularly in birds (Yokoyama et al., 2000; Carvalho et al., 2007). Furthermore, although only some birds express UVS pigments, birds with VS pigments are likely able to detect UV light because SWS1 cone oil droplets and ocular media transmit light in this range (Bowmaker, '80). In pigeons, which are known to have a VS SWS1 pigment, behavioral and electrophysiological experiments have demonstrated UV sensitivity as low as 320 nm (Kawamura et al., '99), and their feathers have peak

reflectance in the UV (McGraw, 2004). Moreover, some passerines do not have UVS SWS1 pigments even though UV colors are likely involved in the courtship display (Andersson and Amundsen, '97; Hunt et al., '98; Alonso-Alvarez, 2004; Coyle et al., 2012; van Hazel et al., 2013). These studies suggest UV pigments are not necessary to perceive UV signals, and perhaps the presence of an SWS1 pigment with maximal absorption in the UV is not as advantageous as previously thought.

Because the functional relevance of wavelength differences among VS-type pigments is poorly understood, and the residues responsible for variation among VS pigments not well characterized, models based on pigeon and chicken may be inappropriate for estimations of visual characteristics in other birds, such as passerines. Instead, we suggest that when neither *in vitro* expression nor MSP of SWS1 is feasible, a mid range VS-type pigment, such as the bowerbird SWS1, may generate a superior estimation.

CONCLUSIONS

This review explores the variability of amino acid residues conferring SWS1 λ_{max} shifts in vertebrates, unique functional qualities inherent to SWS1 pigments, and the strategies used to infer SWS1 spectral characteristics. It is clear that the residues governing spectral shifts between UVS and VS SWS1 in birds are unusually consistent compared to other vertebrate groups, but we emphasize that the lack of mutagenesis studies throughout the diverse avian orders limits our ability to speculate on the roles of spectral tuning sites across the entire class. A valuable addition to the growing body of literature on SWS1 would involve an exploration of the mechanisms mediating variation λ_{max} within VS SWS1. Furthermore, at the structural level, detailed studies of the precise mechanisms stabilizing the deprotonated and protonated Schiff base linkage in SWS1 pigments will provide key insights into the molecular basis of spectral tuning in these pigments. Expanding upon whether an organism has UVS or VS SWS1 by identifying how and where these transitions occurred in evolutionary history, and their adaptive implications, remain important lines of inquiry.

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