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Visual pigments, oil droplets, lens, and cornea characterization in the whooping crane (*Grus americana*)

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Abstract

Vision has been investigated in many species of birds, but few studies have considered the visual systems of large birds and the particular implications of large eyes and long-life spans on visual system capabilities. To address these issues we investigated the visual system of the whooping crane, *Grus americana* (Gruiformes: Gruidae). *G. americana* (an endangered species) is one of only two North American crane species and represents a large, long-lived bird where ultraviolet sensitivity may be degraded by chromatic aberrations and entrance of ultraviolet light into the eye could be detrimental to retinal tissues. To investigate the whooping crane visual system we used microspectrophotometry to determine the absorbance spectra of retinal oil droplets and to investigate if the ocular media (i.e., the lens and cornea) absorbs UV light. *In vitro* expression and reconstitution was used to determine the absorbance spectra of rod and cone visual pigments. The rod visual pigments had wavelengths of peak absorbance (λ_{max}) at 500 nm, while the cone visual pigments λ_{max} values were determined to be 404 nm (SWS1), 450 nm (SWS2), 499 nm (RH2), and 561 nm (LWS), similar to other characterized bird visual pigment absorbance values. The oil droplet cutoff wavelength (λ_{cut}) values similarly fell within ranges recorded from other avian species: 576 nm (R-type), 522 nm (Y-type), 506 nm (P-type), and 448 nm (C-type). We confirm that *G. americana* has a violet-sensitive visual system, although based on the λ_{max} of the SWS1 visual pigment (404 nm) may also have some ability for UV sensitivity.

Introduction

The assortment of photoreceptors in bird retinas is highly conserved across species. Most species of birds that have been investigated have retinas with four spectral classes of single cones, a set of double cones comprised of a principal and an accessory cell, and a rod photoreceptor class (Bowmaker, 1977; Bowmaker et al., 1997; Hart 2004; Hart et al. 2000; Hunt et al., 2009; Wright and Bowmaker, 2000). Moreover, avian cone photoreceptors contain oil droplets within the distal region of the inner segment. These oil droplets are colored due to the presence of carotenoid pigments, ranging in color from clear through pale green to yellow and red. These oil droplets act as long-pass filters, tuning the sensitivity of the underlying visual pigments to longer wavelengths, narrowing the sensitivity function of the underlying photoreceptor, and reducing overlap with adjacent photoreceptor spectral types (Bowmaker, 1977; Bowmaker et al., 1997; Hart 2004; Hart et al. 2000; Hunt et al., 2009).

Typically there are five different classes of oil droplets found in bird retinas, each of which is paired with a particular cone photoreceptor and visual pigment type (Table 1; Bowmaker et al. 1997, Hart et al. 2000; Hart & Hunt 2007; Hart 2001b; Hunt et al. 2009). While visual pigments are characterized by their wavelength of maximal absorbance (λ_{max}), oil droplets are classified by their cutoff wavelength (λ_{cut}) values, which describe the wavelength below which no significant light is transmitted (Lipetz, 1984). Avian rod photoreceptors, containing rhodopsin 1 (Rh1) visual pigments, do not have oil droplets. Long-wavelength sensitive (LWS) visual pigments are either found in single or double cones. LWS single cone oil droplets appear orange to red in color ('R-type'). In double cones, the principal photoreceptor contains an oil droplet that appears colorless, pale green, or yellow ('P-type'; Partridge 1989; Hart 2001a), while the accessory photoreceptor only occasionally contains a small droplet (Hunt et al., 2009). Cones with rhodopsin 2 (Rh2; cones expressing Rh2 opsin genes are designated as middle-wavelength sensitive or MWS) visual pigments contain yellow, 'Y-type', oil droplets. Cones with short-wavelength sensitive type 2 (SWS2) cone visual pigments have clear to pale green, 'C-type', oil droplets that contain carotenoids absorbing strongly below 450nm (Goldsmith et al., 1984; Hart, 2001a; Hart, 2001b; Partridge, 1989). Finally, short-wavelength sensitive type 1 (SWS1) visual pigments are expressed in cone photoreceptors that contain transparent, 'T-type', oil droplets that lack carotenoid pigments and have negligible absorbance across the ultraviolet and visible spectrum.

Many studies have investigated the components of avian visual systems, particularly the spectral characteristics of the visual pigments and oil droplets within the passerines (Hart, 2001b; Hart and Hunt, 2007; Hunt et al., 2009). Based on the type of SWS1 visual pigment expressed, avian visual systems can be classified as either violet sensitive (VS; λ_{max} = 402-426 nm) or ultraviolet sensitive (UVS; λ_{max} = 355-380 nm). To understand the potential functional differences of these two broad visual categories, characterization of additional visual system components are needed. Most significant are the transmission characteristics of the lens and cornea, which can effectively absorb UV light and define the limits of photoreceptor UV sensitivity. In relation to ultraviolet vision, Hart (2001b) hypothesized that smaller birds are more likely to have UVS pigments, possibly either to improve locomotive flight ability or because of the characteristics of unpigmented ocular media allowing for the transmission of enough UV light to be useful for vision only in small eyes. Supporting this hypothesis, Lind et al. (2014) found a strong correlation between eye axial length (a proxy for body size) and ocular media transmission of 24 species of bird, supporting the hypothesis that eye size constrains avian sensitivity to UV light.

Of particular interest are the effects of UV light on visual system capabilities. UV light tends to be more strongly scattered than visible light, reducing contrast. It also is refracted more strongly than visible light by the cornea and lens, and the resulting longitudinal chromatic aberration could further reduce spatial resolution and contrast (Bennett and Cuthill, 1994). Furthermore, UV wavelengths of light can be damaging to retinal tissues, with accumulating effects that have the potential to be severe for long-lived species (Sliney, 2002; Zuclich, 1989). However, many larger, long-lived birds have UV-sensitive visual systems, including UV transmissive ocular media (Carvalho et al., 2011). The question of how large, long-lived birds balance the ecological demands leading to the evolution of UVS with the potential for UV-induced retinal damage remains largely unanswered.

In this study, we characterize the visual system of the whooping crane, *Grus americana* (Linnaeus, 1758). This study represents the first visual system characterization for a species from within the Gruiiformes that includes measuring visual pigment and oil droplet absorbance, as well as examining the lens and cornea for UV absorbance properties. Whooping cranes are large birds that can live up to 40 years in captivity (Wasser & Sherman, 2010). Birds with larger eyes, such as *G. americana*, are predicted to use a violet-sensitive SWS1 visual pigment to

improve image quality by decreasing defocus due to longitudinal chromatic aberration (Hart and Hunt, 2007). Additionally, ocular media that absorbs a large percentage of UV light would reduce the retinal damage due to ultraviolet light over the long life span of this species. As a further protection against retinal damage, corneal and lens materials are predicted to absorb UV light in large, long-lived birds. As large, long-lived birds that have distinct visual behaviors (Cronin et al., 2007) and are subject to intensive conservation efforts, characterizing crane vision contributes to understanding the ecology of this species for management purposes and more broadly provides insight to the ecological and evolutionary forces shaping avian visual systems.

Results

Opsin retinal expression and visual pigment absorbance

Five full-length opsin transcript sequences were isolated from whooping crane retinal RNA. Based on phylogenetic analysis, these transcripts are related to other avian opsin sequences and correspond to the five major vertebrate visual pigment classes: RH1 (1056 bp), RH2 (1068 bp), SWS1 (1044 bp), SWS2 (1029 bp), and LWS (1095 bp) (Fig. 1). Using *in vitro* expression studies, these five opsins form visual pigments that fall within the known variation for bird rod and cone photoreceptors (Fig. 1, Table 1). The four cone visual pigments had peak absorption (λ_{max}) values of 404 nm (SWS1), 450 nm (SWS2), 499 nm (RH2), and 561 nm (LWS) (Fig. 1). The rod visual pigment had a measured λ_{max} of 500 nm (RH1). Based on the λ_{max} of the *G. americana* SWS1 visual pigment (404 nm), the whooping crane visual system can be classified as violet-sensitive (VS). Similar to other birds, violet sensitivity in the whooping crane SWS1 visual pigment corresponds with the presence of amino acid residues Cys86 and Ser90.

Oil droplet, lens and cornea absorptances

We measured the spectral absorbance of 36 oil droplets from a single whooping crane retina. Based on color, as well as the shape and calculated λ_{cut} of the measured absorbance curves, five types of oil droplets were characterized in the *G. americana* retina with λ_{cut} values of 576 nm (R-type), 522 nm (putative Y-type), 506 nm (putative P-type), 448 nm (C-type), and negligible absorptance across all wavelengths (T-type), (Table 2, Fig. 2). The calculated λ_{cut} values for each oil droplet type either fell within values reported for other avian species (R-type, C-type, T-type), or were slightly red-shifted relative to previously reported values (P- and Y-type) (Tables

1, 2). The absorptances of the whooping crane cornea and partial lens tissue were both low (Fig. 3). Relevant to the question of *G. americana* UV sensitivity, both tissues had similar rises in absorptance in the ultraviolet range starting at ~420nm.

Discussion

Visual pigments

Five classes of opsin sequences were isolated from whooping crane retina. The expression of these opsin transcripts demonstrates that whooping cranes have a typical bird visual system, containing four visual pigments used in cone photoreceptor classes, and one visual pigment expressed in rod photoreceptors. The measured λ_{max} from heterologous expression of all of the crane visual pigments fall within known avian absorbance peak variation for each spectral class. Of particular interest is the λ_{max} of the SWS1 visual pigment, where a 404-nm peak absorbance gives the whooping crane a violet-sensitive visual system.

Site 90 is the main amino acid controlling VS/UVS in the SWS1 visual pigments of birds (Wilkie et al. 2000; Yokoyama et al. 2000). Based on ancestral state reconstructions of the three amino acid sites (86, 90, and 93) important to tuning SWS1 visual pigments to be either UVS or VS, avian color vision has shifted between VS and UVS at least 14 times (Ödeen and Håstad 2013). Additionally, violet sensitivity has been hypothesized to be the ancestral state for bird SWS1 visual pigments (Hunt et al. 2009), though this hypothesis is tenuous as only one of the three sites can be reconstructed unequivocally (Ödeen and Håstad 2013). A second site, residue 86, has also been shown to play a role in shifts between VS and UVS visual pigments more broadly in vertebrates. In previous studies of bird SWS1 visual pigment spectral sensitivities, site-directed mutagenesis demonstrated that both Ser86Cys and Ser90Cys mutations generate a UV shift in λ_{max} (Shi and Yokoyama, 2003; Wilkie et al., 2000). Based on sequence data alone, the SWS1 gene from two species from within the Gruiformes – the crowned crane *Balearica pavonina* (Gruidae) and the common coot *Fulica atra* (Rallidae) – are both predicted to have a λ_{max} value of 406 nm based on the an amino acid replacement in only one of these sites, the Cys86 residue (Ödeen and Håstad, 2003). This study represents the first spectral characterization of any crane visual pigments to test these predictions. The whooping crane SWS1 gene sequence contains the same Ser90 / Cys86 amino acid residues as characterized in

other Gruiformes, and the 404 nm λ_{max} matches the predicted values for the crowned crane and common coot extremely well (Ödeen and Håstad, 2003).

Oil droplets

Avian visual systems generally contain five types of oil droplets associated with specific photoreceptor types. We characterized five types of oil droplets from whooping crane data. Three of these types can be clearly classified as the T-type, C-type, and R-type droplets (Table 2). Of these, the C-type and R-type oil droplets exhibit typical avian absorptance curves. When compared to data from other birds, however, the T-type droplet appears to have a relatively high absorptance (~0.2), particularly below 400nm (Fig. 2). It is possible the T-type high absorptance is a measurement artifact of using an MSP beam (2μm) that is slightly larger than the diameter of the droplets being measured. If this high absorptance is real, however, it may indicate there are low levels of carotenoid in the T-type droplet that provide more of a filtering effect than found in other bird visual systems.

The remaining two types represent the Y-type and P-type droplets, which can have very similar λ_{cut} values. Because we were unable to use fluorescence to help unambiguously classify these oil droplet types during spectral characterization (Hart, 2001a), the Y- and P-type λ_{cut} values are putative. Based on λ_{cut} ranges from other avian species and on the function of oil droplets as long-pass filters, we putatively assigned the droplets with λ_{cut} values of 522 nm as Y-type, and the droplets with 506 nm λ_{cut} values as P-type droplets (Table 2, Fig. 4).

The gruiform species that have been studied previously have unique Y-type and P-type oil droplet properties. In general, gruiforms have more intensely pigmented (i.e., more orange) Y-type droplets than those found in most other birds (Hart, 2001a). Although the spectral transmittance characteristics of single cone oil droplets tend to vary little across the retina, in the dusky moorhen *Gallinula tenebrosa* (Gruiformes, Rallidae) the P-type droplets varied in color across the retina, from almost as orange as the Y-type oil droplets to a pale yellow (Hart, 2001a). The whooping crane has P-type and Y-type oil droplets that have similar absorbance characteristics based on our putatively assigned λ_{cut} values. Additionally, the most abundant oil droplet type across an avian retina is the P-type, on average representing 29.0-55.5% of the photoreceptors (Hart, 2001a). Within the dusky moorhen (*Gallinula tenebrosa*; Gruiformes) retinal oil droplet composition includes 20.2% Y-Type and 33.0% P-Type (Hart 2001). Based

on the absorbance similarities of our Y- and P-type droplet classes (Table 2), P-type λ_{cut} variations across the retina, as well as retinal oil droplet composition differences, it is also possible that our putatively assigned droplet types either represent P-type droplets from different retinal regions, or represent mixtures of Y-type and P-type droplets. Because of the limitations of working with an endangered species, particularly with acquiring fresh retinal tissues, our sampling of oil droplet types in the whooping crane was limited. If material becomes available, future studies should characterize the yellow and pale oil droplet more thoroughly, and look for variations in λ_{cut} values in different retinal regions.

Cornea & Lens

Because the T-type oil droplet class has negligible absorption, the sensitivity of the SWS1 photoreceptor is determined by the λ_{max} of the visual pigment and the absorbance of the ocular media (Hart, 2001b). This is similar to the findings of Lind et al. (2014), who modeled the visual system of 38 bird species based on visual pigment λ_{max} and ocular media transmittance. This study found that color discrimination in bright light is mostly dependent on the visual pigment (UVS or VS), regardless of ocular media characteristics, hypothesizing that ocular media spectral tuning is mainly relevant for detecting weak UV signals. At the low end of VS bird vision, SWS1 sensitivity overlaps with the upper part of the UV range (e.g. 380 nm), as is the case with the 404 nm SWS1 whooping crane visual pigment. Consequently, a limited UV sensitivity may be possible despite cornea and lens properties, given whooping cranes are mostly white birds that are active during the day when reflectance would be maximized in the UV portion of the spectrum.

Birds with VS visual systems tend to have ocular media with longer-wavelength transmission (measured as $\lambda_{T0.5}$, the wavelength at half the measured transmission maximum) values, ranging from 335-378 nm, while those with UVS have values ranging from 316-343 nm (Hart and Hunt, 2007). Much of the ocular media absorbance properties come from the lens, with the cornea contributing little to overall ocular media UV absorbance, therefore our corneal absorbance data are not surprising. The absorbance measured from the whooping crane lens is more surprising, particularly in the UV part of the spectrum (Fig. 3). Because our measurements were made on pieces of dissected lens tissue, we have not fully characterized the intact lens UV absorbance characteristics and we predict that intact whooping crane lenses are most likely much

more UV absorbant. The drop in absorptance below ~375nm may be due to leaching of some of the lens pigments from lens pieces, or due to some uncharacterized fluorescence corrupting scans. Additionally, many birds have multifocal lenses (Lind and Kröger, 2008), and although the focal properties within Gruiformes species are unknown, our measurements may have missed lens areas important to UV absorbance. Despite these potential artifacts, the rise in absorptance in the blue portion of the lens spectrum suggests that the whooping crane intact lens most likely has a $\lambda_{T0.5}$ value somewhere in the 380-410nm range, consistent with other birds with VS visual systems.

Similar to the whooping crane, the visual systems of several other large birds have been characterized as VS, including the turkey (*Meleagris gallopavo*, Hart et al. 1999), peafowl (*Pavo cristatus*, Hart 2002), and ostrich (*Struthio camelus*, Wright and Bowmaker, 2001). Other larger bird species with violet-sensitive visual systems have expected $\lambda_{T0.5}$ values in the longer-wavelength ranges of UV light, between 358 and 370 nm (Hart et al. 1999; Hart 2002; Wright and Bowmaker, 2001). Interestingly, of those species with SWS1 visual pigment λ_{max} values that are similar to those we found in the whooping crane, there is a large variation in ocular media transmission (Ostrich: SWS1 λ_{max} = 405 nm, ocular media $\lambda_{T0.5}$ = 377 nm; wedge-tailed shearwater: SWS1 λ_{max} = 406 nm, ocular media $\lambda_{T0.5}$ = 335 nm) (Hart and Hunt, 2007). One possibility for this variation is related to eye size; the wedge-tailed shearwater eye is smaller than the ostrich, reducing chromatic aberration and thus permitting an ocular media with a shorter $\lambda_{T0.5}$. However, the variation in ocular media transmission in relatively large eyes also demonstrates that long optical paths do not necessarily absorb the UV portion of the spectrum. This suggests that there may be some fine-scale tuning of visual system UV sensitivity in the absorbance properties of the ocular media based on the visual ecology of the species. Despite not having fully characterized lens UV absorbance properties, the possibility exists of limited UV vision, particularly in bright light environments, in the whooping crane based on a SWS1 visual pigment shifted towards UV wavelengths. This suggests there may be a role for functional UV sensitivity in whooping crane visual ecology.

Visual System Implications for Grus americana

Retinal damage due to long-term UV exposure is a potential issue for long-lived birds like the whooping crane. Therefore, to avoid retinal damage, long-lived birds might be expected to have

UV-absorbing ocular media, making UV-sensitive photoreceptors superfluous (Carvalho et al., 2011). Additionally, studies of other avian visual systems found a strong correlation between eye size and the ocular media transmittance, with larger eyes having longer $\lambda_{T0.5}$, the wavelength at half the measured transmission maximum (Lind et al., 2014). The whooping crane visual system in part matches these predictions by having a violet sensitive SWS1 visual pigment.

Within birds with VS visual pigments there is variation in ocular media transmittance (Lind et al. 2014). If whooping crane lenses have low UV absorbance, mechanisms to repair UV light damage would be needed, as with other long-lived birds having UV sensitive visual systems (e.g. Psittaciformes) (Carvalho et al., 2011). It is also possible that the extremely violet-sensitive SWS1 visual pigment is a compromise between the longitudinal chromatic aberration that would result from transmission of short-wavelength UV light (necessary to excite UVS pigments) and the need to detect UV signals, perhaps in plumage. Nearly all white feathers reflect significant amounts of UV (Eaton and Lanyon, 2003; Mullen and Pohland, 2008), and the ability to detect some UV light may contribute to the overall brightness of the reflected light from the white *G. americana* feathers. The violet sensitive *G. americana* visual system, with a SWS1 absorbance peak at 404 nm, would be capable of detecting signals in the UV range.

Summary

In this study we measured the spectral characteristics of the *Grus americana* visual pigments, oil droplets, cornea, and partial lens tissue. The whooping crane contains a typical set of avian visual pigments and oil droplets, and as predicted a violet-sensitive visual system. Further work characterizing the distribution of oil droplets, as well as the potential for variation in oil droplet absorbance, across the retina would further contribute to the overall understanding of whooping crane visual systems. Measurements of intact corneal absorbance indicate this tissue does not contribute to UV light filtering in the whooping crane eye, while our measurements of lens pieces suggests the presence of some near-UV absorbance in the lens as a whole. The presence of a short SWS1 visual pigment in a diurnal animal suggests that whooping cranes have a visual system capable of detecting UV light, warranting future studies of whooping crane plumage reflectance. The difference in ocular media absorbance characteristics among species with VS visual systems suggests that rather than tuning either visual pigments or oil droplets, some birds may fine-tune UV sensitivity with ocular media absorbance.

Materials and Methods

Tissue acquisition and storage

Tissues were collected from a single captive individual of the whooping crane, *Grus americana*, from a breeding colony established at the USGS Patuxent Wildlife Research Center, MD, USA. The animal was euthanized due to circumstances unrelated to this study, and eye and muscle tissues were collected immediately after euthanization. Because the whooping crane is an endangered species and tissue from additional individuals was not available, retinal tissue from one eye was used to determine the spectral characteristics of the oil droplets, cornea, and lens, while the other was used for molecular techniques to isolate and characterize the opsin genes. Collected tissues were dissected from the bird and either placed on ice until use (for spectral measurements), or immersed in either ethanol (muscle tissue) or RNAlater (retinal tissue; Qiagen, Valencia, CA, USA) and stored at -80°C for use in DNA or RNA extractions for molecular studies.

Characterization of opsins expressed in the retina and phylogeny reconstruction

Retinal tissue was dissected out of a single, fresh crane eye and used in standard RNA extraction protocols (TRIzol, Life Technologies, Carlsbad, CA, USA). Retinal total RNA was used for first strand synthesis with an oligo(dT) primer and Superscript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA). The resulting cDNA was used for 3' RACE procedures with degenerate primers based on alignments of published bird and squamate opsin sequences for each class of vertebrate visual pigments (RH1, RH2, LWS, SWS1, SWS2; Table 3). Positive double stranded RT-PCR products were cloned using the pGEM-T Easy vector system (Promega, Madison, WI, USA), and individual clones screened via sequencing with vector primers. Once a piece of each gene was isolated, if the 5' end of the transcript was missing 5' RACE was performed using 3' gene specific primers using the 5'/3' RACE kit, 2nd generation (Roche, Madison, WI, USA). Full-length transcripts were confirmed by sequencing with a proofreading reverse transcriptase (AccuScript Hi-Fi Reverse Transcriptase, Agilent Technologies, Santa Clara, CA, USA) and polymerase (PrimeSTAR HS DNA polymerase, Takara, Japan) using gene specific primers designed to the 5' and 3' ends of each transcript (Table 3). All PCR products were sequenced by Genewiz, Inc. (Germantown, MD, USA). Full

length opsin transcript sequences have been deposited to the NCBI Genbank database (**accession numbers provided upon acceptance**).

To place the characterized opsin sequences in an evolutionary context, whooping crane transcripts were aligned with publicly available opsin sequences from birds including *Corvus macrorhyncos* (**RH1**: BAJ05379), *Serinus canaria* (**RH1**: CAB91997; **RH2**: CAB91995; **SWS1**: CAB91993; **SWS2**: CAB91994; **LWS**: CAB91996), *Taeniopygia guttata* (**RH1**: AAF63461; **RH2**: NP_001070164; **SWS1**: AAF63463; **SWS2**: NP_001070165; **LWS**: NP_001070170), *Columba livia* (**RH1**: AAD32241; **RH2**: AAD32242; **SWS1**: AAD38034; **SWS2**: AAD38035; **LWS**: AAD38036), *Gallus gallus* (**RH1**: P22328; **RH2**: P28683; **SWS1**: P28684; **SWS2**: NP_990848; **LWS**: NP_990771), *Melopsittacus undulatus* (**RH1**: AAC41247; **RH2**: AAC41246; **SWS1**: CAA72483), *Anas platyrhynchos* (**RH1**: AAC41245; **RH2**: EOA96196), *Ptilonorhynchus violaceus* (**SWS2**: AFK82745; **LWS**: AFK82727), *Chlamydera nuchalis* (**SWS2**: AFK82748; **LWS**: AFK82730), *Chlamydera maculata* (**SWS2**: AFK82747; **LWS**: AFK82729), *Ailuroedus crassirostris* (**SWS2**: AFK82743; **LWS**: AFK82725), *Scenopoeetes dentirostris* (**SWS2**: AFK82744; **LWS**: AFK82726), *Calyptorhynchus latirostris* (**SWS1**: ADG96042), *Cacatua galerita* (**SWS1**: ADG96044), *Eolophus roseicapillus* (**SWS1**: ADG96043), *Barnardius semitorquatus* (**SWS1**: ADG96041), *Platycercus elegans* (**SWS1**: ADG96036), *Cyanistes caeruleus* (**SWS1**: AAP30082), *Palacrocorax carbo* (**SWS1**: ABS86975), *Spheniscus humboldti* (**SWS1**: CAC20913), *Sericulus chrysocephalus* (**LWS**: AFK82728). Amino acid sequences were aligned using MAFFT (Katoh et al., 2005; Katoh et al., 2002) and the resulting alignment used to estimate phylogenetic relationships and node confidence as bootstrap values using RAxML (Stamatakis, 2006; Stamatakis and Hoover, 2008). Based on previously published phylogenies of opsin relationships (Porter et al., 2012), the phylogeny was rooted using avian (*Gallus gallus*: ACX32474; *Taeniopygia guttata*: NP_001266194), anuran (*Xenopus laevis*: NP_001165363), and squamate (*Anolis carolinensis*: ACX32472) sequences from the clade designated 'vertebrate ancient (VA) opsins', which are evolutionarily basal to the vertebrate visual pigments (Philp et al. 2000; Porter et al., 2012).

Visual Pigment Heterologous Expression, Purification and Spectral Analysis

To characterize visual pigment spectral absorbance *in vitro*, each full-length opsin gene was first cloned into the mammalian expression vector pMT3 and appended with the last 15 amino acids

of bovine rhodopsin (Franke et al., 1988; Oprian et al., 1987). Because we initially had difficulty characterizing the 5' end of the LWS opsin transcript (~30 bp), a construct consisting of the first 16 bp from the *Columbia livia* LWS opsin followed by the near-full length whooping crane LWS opsin was created for *in vitro* expression. Based on the known function of the opsin N-terminal region in proper protein localization but not in spectral tuning, this construct allowed us to characterize the whooping crane LWS visual pigment absorbance. All constructs were expressed transiently in HEK 293A cells using Turbofect transfection reagent (Thermo Scientific, Waltham, MA, USA). Briefly, 10 µg of plasmid DNA was mixed with 20 µl of Turbofect and incubated in 1.0 mL Dulbecco's Modified Eagle Medium (Life Technologies, Carlsbad, CA, USA) for 20 minutes and added to an 80% confluent 100 mm x 20 mm plate of HEK 293A cells. Cells were harvested at 48 h post transfection. Protein expression was confirmed by Western Blot analysis using anti-1D4 primary antibody and an alkaline phosphatase conjugated secondary antibody (data not shown). Blots were visualized after incubation with AttoPhos fluorescent substrate (Promega, Madison, WI, USA) on a Storm 840 molecular imager (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

After confirmation that each construct expressed opsin in the HEK 293A cells at sufficient levels, proteins were purified for spectral characterization. All purification steps were performed under dim red light at 4°C. Thirty 100 mm x 20 mm plates of transiently transfected HEK 293A cells were harvested and washed once in 1x PBS. Before solubilization, photopigments were reconstituted in 5 mL of 10 mM MES/150 mM NaCl buffer (pH 6.0) containing 40 µM 11-*cis* retinal for 1 hour. Membranes were solubilized in 10 mM MES/150 mM NaCl buffer (pH 6.0), 1% n-Dodecyl-β-D-maltoside (DM), 0.2 mg/ml PMSF for 2.5 hours and spun in a clinical centrifuge to pellet debris. Homogeneous protein supernatant was incubated with anti-1D4 Sepharose column matrix for 16 hours (Wilkie et al. 2000). The photopigment/column matrix complex was washed 10 times with 10 mM MES/150 mM NaCl buffer (pH 6.0), 0.1% DM to remove excess chromophore. Photopigment was eluted from column matrix in 0.1% DM, 80 µM 1D4 peptide. Eluates were concentrated to ~250µL using Amicon Ultra 30K MWCO centrifugal filters (Millipore, Billerica, MA, USA). Spectrophotometric measurements were taken from 240 to 700 nm in 1-nm increments with a Hitachi U3300 UV-Vis Spectrophotometer.

Microspectrophotometry: Lens, cornea and oil droplet absorbance

Microspectrophotometry was used to measure the spectral absorbance of photoreceptor oil droplets and to investigate the UV absorbance of intact cornea and partial lens tissue of *G. americana* eyes. All tissues for absorbance measurements were collected from a single eye. For all measurements (lens, cornea, and oil droplets), a 2 μm beam was placed in the tissue of interest (oil droplet, lens, or cornea) and absorbance was measured at 1-nm intervals from 350 to 700 nm. For oil droplet measurements, multiple droplets of each class, as determined by color to the human eye, were measured from a single retina (Table 2). The best oil droplet absorbance spectra for each color class (n=3 to 9) were converted to absorptance, averaged, and used to calculate the λ_{cut} values for each drop type, defined as the wavelength of the intercept at the value of maximum measured absorptance by a line tangent to the absorptance curve at half-maximum measured absorptance (Lipetz, 1984). Although the best way to characterize ocular media transmission is *in situ* measurement of lens, cornea, and vitreous humor spectral properties (Lind et al. 20014), the opportunistic availability of the whooping crane tissue and limited sample size (i.e. 1 animal, 2 eyes) made it impossible to prepare the materials required for this technique before the tissue became unusable. Therefore, we used MSP to investigate the absorbance properties of the isolated cornea and pieces of the lens tissue, as a measure of whether or not whooping crane ocular media contained any significant UV absorbance. For these measurements, the cornea and lens were dissected from a single eye. Corneal measurements were made on whole cornea tissue, sandwiched in 1X PBS between two UV transmissive coverslips. For lens measurements, pieces of lens tissue were used in the same setup (i.e. in 1X PBS held in place between UV transmissive coverslips). For both lens and corneal measurements, the best absorbance measurements (n= 4 and 7, respectively) were averaged and converted to absorptance. Typically lens and corneal spectral characteristics are described in terms of ocular transmission ($\lambda_{T0.5}$, the wavelength at half the measured transmission maximum; (Emmerton et al., 1980). However, given the methods used to characterize absorbance, and the low UV absorbance measured in both tissue types, calculating $\lambda_{T0.5}$ was not possible.

Symbols / Abbreviations

λ_{max} - wavelength of peak absorbance

λ_{cut} - oil droplet cutoff wavelength

$\lambda_{T0.5}$ - the wavelength at half the measured transmission maximum

SWS1 – vertebrate cone short-wavelength sensitive type 1 visual pigments

SWS2 – vertebrate cone short-wavelength sensitive type 2 visual pigments

MWS – cone photoreceptors with middle wavelength-sensitivity

RH2 – vertebrate middle wavelength-sensitive cone visual pigments

RH1 – vertebrate rod visual pigments

LWS - vertebrate cone long-wavelength sensitive type visual pigments

R-type – red-type avian oil droplets

P-type - pale-type avian oil droplets

Y-type - yellow-type avian oil droplets

C-type - clear-type avian oil droplets

T-type - transparent-type avian oil droplets

UV - ultraviolet

UVS – ultraviolet sensitive

VS – violet sensitive

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Competing Interests

The authors declare no competing financial interests.

Author Contributions

M.L.P. assisted with the supervision of the molecular characterization of the opsin genes and the spectral characterization of the oil droplets, analyzed the data, and wrote the manuscript. A.C.N.K. and L.S. characterized the opsin sequences, C.H. measured the oil droplet spectra, and R.M. and E.C. performed heterologous expression experiments. G.H.O. provided access to the whooping cranes and dissected out the appropriate tissues. P.R.R. and T.W.C. supervised the project and provided all other laboratory resources.

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Figure 1. **Opsin sequence diversity and corresponding visual pigment absorbance spectra from *Grus americana* retinal tissues.** The maximum likelihood phylogeny of avian opsin sequences is rooted using representative avian, anuran, and squamate using sequences from the clade designated 'vertebrate ancient (VA) opsins', which are evolutionarily basal to the vertebrate visual pigments. Numbers above branches are bootstrap proportion nodal support values. The major vertebrate opsin clades have been indicated: rhodopsin 1 (RH1), rhodopsin 2 (RH2), short-wavelength sensitive 1 (SWS1), short-wavelength sensitive 2 (SWS2), and long-wavelength sensitive (LWS). The *G. americana* visual pigment spectra are plotted next to the corresponding opsin group for each sequence. For each opsin sequence, the normalized visual pigment absorbance curve is plotted in grey, and the template fit is in black. The calculated wavelength of maximum absorbance based on template fitting is indicated in the upper right corner of each spectral plot.

Figure 2. **Whooping crane oil droplet absorptance.** A) Baseline corrected absorptance of each class of whooping crane oil droplet. Each droplet is plotted as follows: red = R-type, yellow = Y-type, green = P-type, blue = C-type; grey = T-type. B) Histogram of λ_{cut} values for all measured oil droplets with the exception of the T-type, colored as in panel A. Inset is a photograph taken through the microspectrophotometer showing the colors of whooping crane retinal oil droplets: R = R-type, P/Y = P- and Y-Type droplets, C = C-type, and T = T-type droplets.

Figure 3. **Whooping crane cornea (grey) and lens (black) absorptance.**

Figure 4. **Overlay of visual pigment (solid lines) and oil droplet (dotted lines) absorptance curves, and calculated sensitivity of the four main cone types.** A) SWS1 and SWS2 visual pigments, and C-type oil droplet; B) Rh2 visual pigment and Y-type oil droplet; C) LWS visual pigment with R-type and P-type oil droplets; D) Normalized sensitivity of the four main cone photoreceptors based on visual pigment absorbance and oil droplet transmittance.

Table 1. Typical avian oil droplet photoreceptor association and wavelength cutoffs (data from Bowmaker et al. 1997, Hart et al. 2000; Hart & Hunt 2007; Hart 2001b; Hunt et al. 2009).

Type	Color	Wavelength cutoff (λ_{cut})	Associated Photoreceptor	Range of peak absorbances (λ_{max})
---	No droplet	---	Rh1 rods	500-510 nm
P-type	Pale	460-498 nm	Double cones	543-571 nm
R-type	Red	514-586 nm	LWS cones	543-575 nm
Y-type	Yellow	490-516 nm	MWS cones	497-510 nm
C-type	Clear	392-449 nm	SWS2 cones	427-463 nm
T-type	Transparent	<350 nm	SWS1 cones	355-380 nm (UVS) 400-426 nm (VS)

Table 2. Whooping crane visual pigment wavelength of peak absorbance (λ_{max}) and oil droplet cutoff wavelength (λ_{cut}) in nm. λ_{cut} values are followed in parentheses by the number of oil droplet scans included in each average. The λ_{max} of the double cones (in grey) is inferred based on expression patterns of the LWS visual pigment in other bird species.

Photoreceptor	LWS	MWS	SWS1	SWS2	Double-Cones	Rods
λ_{max} (nm)	561	499	404	450	561	500
Oil droplet	R-type	Y-type	T-type	C-type	P-type	---
λ_{cut} (nm)	576 (9)	522 (3)	--- (13)	448 (8)	506 (3)	---

Table 3. Primer sequences used to characterize whooping crane opsin transcripts.

Primer	Sequence (5'->3')
Degenerate 3' RACE primers	
WC_RH1/2deg	ATGAAYGGGACRGARGGBRTCAATTTT
WC_SWS1_deg	ATGTCGCRYGASGARGAGTTYTACCTKTT
WC_SWS2_deg	ATGCMGARGSCSCGKGAG
WC_LWS_deg	CGICGICGICAYGAIGAYGARGA
5' RACE primers	
WC_LWSR1075	CTACGCGGGCGCCACGGAGGAGCT
WC_LWSR601	GGCAGAGACGGTGTAGCCCTCGATG
WC_LWSR550	CGAGGTCTGGTTGATGACGCTGAT
WC_LWSR445	CAGACCACGAACCACCGCTCCCA
WC_LWSR371	GGTGTAGCCCTCGATGACGCAGAGC
Gene specific primers	
WC_RH1F	ATGAACGGGACAGAAGGCCAAGACTT
WC_RH1R	TTACAGCCTTGTTCCCAGGGTTCC
WC_RH2F	ATGGATATCTGCAGAATTCGGCTTT
WC_RH2R	CTACGCAGGAGAGACCTGGCTGGT
WC_LWSR	CTACGCGGGCGCCACGGAGGAGCT
WC_SWS1F	ATGTCGGGTGACGAGGAGTTTTACC
WC_SWS1R	TCAGCTGGGGCTGACCTGGCT
WC_SWS2F	ATGCTCCCCGACGACTTCTACATCCCC
WC_SWS2R	CTAGACCTGGGTGGCCTGCGAGGAGGC