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Owls lack UV-sensitive cone opsin and red oil droplets, but see UV light at night: Retinal transcriptomes and ocular media transmittance

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ABSTRACT

Most diurnal birds have cone-dominated retinae and tetrachromatic colour vision based on ultra-violet/violet-sensitive UV/V cones expressing short wavelength-sensitive opsin 1 (SWS1), S cones expressing short wavelength-sensitive opsin 2 (SWS2), M cones expressing medium wavelength-sensitive opsin (RH2) and L cones expressing long wavelength-sensitive opsin (LWS). Double cones (D) express LWS but do not contribute to colour vision. Each cone is equipped with an oil droplet, transparent in UV/V cones, but pigmented by carotenoids: galloxanthin in S, zeaxanthin in M, astaxanthin in L and a mixture in D cones. Owls (Strigiformes) are crepuscular or nocturnal birds with rod-dominated retinae and optical adaptations for high sensitivity. For eight species, the absence of functional SWS1 opsin has recently been documented, functional RH2 opsin was absent in three of these. Here we confirm the absence of SWS1 transcripts for the Long-eared owl (*Asio otus*) and demonstrate its absence for the Short-eared owl (*Asio flammeus*), Tawny owl (*Strix aluco*) and Boreal owl (*Aegolius funereus*). All four species had transcripts of RH2, albeit with low expression. All four species express all enzymes needed to produce galloxanthin, but lack CYP2J19 expression required to produce astaxanthin from dietary precursors. We also present ocular media transmittance of the Eurasian eagle owl (*Bubo bubo*) and Short-eared owl and predict spectral sensitivities of all photoreceptors of the Tawny owl. We conclude that owls, despite lacking UV/V cones, can detect UV light. This increases the sensitivity of their rod vision allowing them, for instance, to see UV-reflecting feathers as brighter signals at night.

1. Introduction

The majority of birds are diurnal and have adaptations for tetrachromatic colour vision and high visual acuity in bright light. Their eye morphology, receptor types and visual pigment spectral tuning are quite uniform: cone photoreceptors largely outnumber rod photoreceptors that are used for vision in dim light (Hart, 2001b). Colour vision is based on four types of single cones expressing opsin-based visual pigments most sensitive to ultraviolet or violet (UV/V cones; short wavelength-sensitive opsin 1, SWS1), blue (S cones; short wavelength-sensitive opsin 2, SWS2), green (M cones; medium wavelength-sensitive opsin, RH2) and red (L cones; long wavelength-sensitive opsin, LWS) light (Hart & Hunt, 2007). In addition, birds have double (D) cones expressing LWS opsin and thought to mediate achromatic vision. All cones (with the common exception of accessory members of double cones) are equipped with oil droplets, in all types except UV/V cones pigmented with carotenoids that narrow the spectral sensitivity and improve colour vision abilities (Olsson, Wilby, & Kelber, 2016; Vorobyev, 2003), at the cost of absolute sensitivity (e.g. Olsson, Lind, & Kelber, 2015). Rods express the rod opsin RH1, have large outer segments and no oil droplets (Mordeshian & Fain, 2017).

Some bird groups, however, have changed lifestyle and are most active during dawn and dusk, or at night. As an adaptation to flightless nocturnal habits, kiwis (*Apteryx* sp.) have reduced eye size and rely more on other sensory cues for foraging (Martin, Wild, Parsons, Kubke, & Corfield, 2007), whereas nocturnal species such as oilbirds and other

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caprimulgiforms have evolved large, highly sensitive eyes to aid their areal foraging (Martin, Rojas, Ramírez, & McNeill, 2004, Rojas, Ramírez, McNeil, Mitchell, & Marín, 2004). The most iconic nocturnal birds are owls (Strigiformes: Strigidae and Tytonidae; Martin, 1990, Martin, 2008), even though this group also includes species with more diurnal activity, such as the Snowy owl (*Bubo scandiacus)* (Boxall & Lein, 1989), and some species with different activity patterns in different populations. Barn owls (*Tyto alba*), for instance, are active in bright light in Great Britain (Bunn, 1972; Dunlop, 1911), but considered strictly nocturnal in other regions.

As adaptations to nocturnal vision, owls have eyes with large apertures and relatively short focal lengths, allowing for bright retinal images Walls, 1942), rod-dominated retinae (Fite, 1973; Martin, 1990; Oehme, 1961; Walls, 1942), and very long rod outer segments (Oehme, 1961). Barn owls and Great horned owls (*Bubo virginianus*) have among the highest absolute visual sensitivities reported among animals (Fite, 1973; Harmening, Nikolay, Orlowski, & Wagner, 2009); barn owls can resolve gratings of 1 cycle/degree in dim starlight (10^{-5}cd m^{-2}; Orlowski, Harmening, & Wagner, 2012). Such high sensitivity results from a rod-dominated retina, with 93% rods and only 7% cones (Fite, 1973), similar to other owl species (Oehme, 1961).

Adaptations to dim light vision are also expected in the efficiency of phototransduction, specifically in rods. Wu et al. (2016) found positive selection in five genes coding for proteins in the phototransduction pathway of rods, specifically those involved in the activation of phototransduction, pigment recovery and adaptation of the photoresponse.

In addition to having low overall cone densities, owls seem to also have lost cone opsins. Several recent studies (Borges et al., 2015; Wu et al., 2016; Hanna et al., 2017) have reported the absence of SWS1 opsin expression in several species of owls, and the absence of RH2 expression in two species of the genus *Tyto*. Ödeen and Håstad (2003, 2013) and Anders Ödeen (personal communication) sequenced SWS1 opsin genes from genomic DNA of a large number of bird species including other nocturnal birds such as the European nightjar (*Caprimulgus europaeus*), but not owls. The lack of SWS1 transcripts is consistent with results by Bowmaker and Martin (1978), who determined cone spectral sensitivities of a Tawny owl (*Strix aluco*) using microspectrophotometry (MSP). They found cone outer segments with only three different absorption peaks: 463 nm (indicative for S cones), 503 nm (M) and 555 nm (L) as well as rods absorbing maximally at 503 nm. Jacobs, Crognale, and Fenwick (1987), using flicker photometry, only confirmed the 555 nm cone pigment for the Great horned owl. Although on their own each of these studies cannot prove the absence of SWS1 in owls, together these various lines of evidence provide strong support for the absence of the SWS1 gene and S cones in owls.

The loss of SWS1 opsin seems to contradict observations on owl visual ecology: Eurasian eagle owls (*Bubo bubo)*, for instance, expose a white, ultraviolet-reflective badge of feathers on the throat while calling at night (Penteriani, Delgado, Alonso-Álvarez, & Sergio, 2007a), and their begging fledglings signal with white mouth feathers (Penteriani et al., 2007b). Whether or not owls can perceive ultraviolet light, ultimately depends on the transmittance of their ocular media, and a previous study has shown that ocular media of four owl species transmit considerably more UV light than those of diurnal raptors (Fig. 3e in Lind, Mitkus, Olsson, & Kelber, 2014).

Adaptations to higher sensitivity are also found in the cones. Cone oil droplets absorb a substantial part of the light that reaches the retina (Wilby & Roberts, 2017) reducing the sensitivity of cone vision. The oil droplets of S, M and L cones contain galloxanthin, zeaxanthin and astaxanthin, respectively, whereas D cone oil droplets contain a mixture of pigments (Goldsmith, Collins, & Licht, 1984; Toomey et al., 2015, 2016). Birds take up zeaxanthin with food, but produce the other carotenoids by modifying zeaxanthin in enzymatic pathways (Toomey et al., 2015, 2016). BCO2 (beta-carotene oxygenase 2), RDH12 (retinol dehydrogenase 12), and RETSAT (retinol saturase) are sufficient to produce galloxanthin and dihydrogalloxanthin, which pigment the S cone oil droplets, from zeaxanthin, and the conversion to astaxanthin that pigments L cone oil droplets (Brush, 1990; Goodwin, 1984; Koch, Hill, & McGraw, 2016) is catalysed by the cytochrome P450 enzyme CYP2J19 (Lopes et al., 2016).

The oil droplets of owls have been reported to be larger (Oehme, 1961), but less pigmented than those of other birds (Bowmaker & Martin, 1978; but see Yew, Woo, & Meyer, 1977); both factors potentially contribute to higher sensitivity of the cones (Wilby & Roberts, 2017). In several owl species they occur in smaller numbers (Bowmaker & Martin, 1978; Muntz, 1972) or are completely absent (Erhard, 1924; Yew et al., 1977). Bowmaker and Martin (1978) did not find intact cones with red oil droplets in the Tawny owl although long wavelength-sensitive cone outer segments were the most frequently recorded in their MSP study. They concluded that these outer segments either belonged to D cones or, alternatively, L cones of the Tawny owl lacked red oil droplets. Recently, Hanna et al. (2017) have reported a frameshift mutation in the gene coding for the enzyme CYP2J19 of the Spotted owl (*Strix occidentalis*). For the same gene, Emerling (2018) also found several loss-of-function mutations in the Barn owl and evidence for pseudogenisation in the Long-eared owl (*Asio otus*).

The objective of this study is to expand the knowledge on nocturnal adaptations of owl vision by: (1) characterizing retinal transcriptomes of four species and identifying genes in the phototransduction pathway, (2) analysing owl opsin genes, specifically the presence of the SWS1 opsin gene, (3) investigating whether genes in the phototransduction pathway of owls evolved under positive or purifying selection, (4) analysing the presence of enzymes in the pathways of oil droplet carotenoid synthesis, and (5) describing the transmittance of ocular media of owls for ultraviolet light.

2. Methods

2.1. Owl specimen

One eye of one individual of the following four species of owls (Strigidae) were used for transcriptome characterizations: Tawny owl (*Strix aluco*), Boreal owl (*Aegolius funereus*), Short-eared owl (*Asio flammeus*) and Long-eared owl (*Asio otus*). The short-eared owl hunts in open habitat, mostly at night but is known to also be crepuscular (Calladine, Garner, Wernham, & Buxton, 2010; Reynolds & Gorman, 1999), whereas the three other species are mainly nocturnal. The tawny owl is territorial throughout the year, and Long-eared owl and Boreal owl have also been reported as being territorial. The tawny owl prefers woodland whereas the latter two species prefer open habitats (Martin, 2008). The Short-eared owl is more frequently active in daylight than the other species.

One eye of the Short-eared owl, the Long-eared owl and the Boreal owl, one eye each of two Tawny owls, and one eye of a Eurasian eagle owl (*Bubo bubo*) were used to measure the ocular media transmittance. Eyes were collected from wild owls of unknown sex that were severely injured and had to be euthanized by a bird rescue station in southern Sweden. All birds were euthanized during daytime.

2.2. Retinal tissue collection and RNA isolation

Eyes were enucleated, retinae excised rapidly and retinal quadrants transferred into 2 ml RNA-later (Ambion) and stored at −80 °C until further processing. RNA was extracted using the RNeasy mini kit (Qiagen) and DNase-treated following the manufacturer's protocols. Quality and quantity of the RNA was assessed on a NanoDrop 2000 (Thermo Scientific) instrument by determination of relative absorbance at 260 and 280 nm. RNA integrity was established using a 2100 Bioanalyzer (Agilent).

2.3. Reverse transcription and PCR using SWS1 specific primers

The RNA extracted from one retina quadrant of Tawny owl and Boreal owl was reverse transcribed to cDNA using a standard kit (QuantiTect Reverse transcription kit, Qiagen). PCRs were set up with primers that had been shown to amplify the SWS1-opsin gene in earlier studies on birds (Ödeen and Håstad, 2003, 2013; Bloch, 2016). The primers designed to amplify the SWS1 gene in 22 species of New and Old World warblers from Bloch et al. (2015) were used according to protocols described in that study.

2.4. cDNA library construction

One sequencing library per species was constructed using TruSeq Stranded mRNA Library Prep Kit (Illumina) according to the manufacturer's instructions. The four libraries were individually tagged with the NEXTflex™ RNA-Seq Barcode adapter set (Bio Scientific) where barcode adapter 17 (GTAGAG) was used for Tawny owl, 18 (GTCCGC) for Boreal owl, 20 (GTGGCC) for Short-eared owl and 21 (GTTTCG) for Long-eared owl. The libraries were sequenced in multiplex in one 300-bp paired-end run on an Illumina MiSeq platform (at the DNA Sequencing Facility, Department of Biology, Lund University) using the MiSeq Reagent Kit V3 (Illumina, cat no. MS-102-3003).

2.5. Data filtering and de novo assembly

Raw reads from the sequencing were quality-checked with FastQC v0.11.5 (Andrews, 2010). They were then pre-processed with Trimmomatic v0.36 (Bolger, Lohse, & Usadel, 2014) to trim low quality bases for reads and remove any remaining sequencing adapters, according to preloaded adapters in ILLUMINACLIP. Leading and trailing low-quality bases were removed with a minimum of Phred score 30 and the reads were scanned with a sliding window of 8 bp, cutting when the average quality per base dropped below Phred score 20. A minimum read length of 100 bp was also used. The Tawny owl library contained a proportion of only poly-A-tail sequences. These were removed with a custom script written in Perl v5.18.2. The trimmed reads were used for *de novo* assembly, performed using Trinity software v2.3.2 (Grabherr et al., 2011). Default settings were used except for "min_kmer_cov" set to 2, meaning that only kmers with a $\geq$ 2x coverage were used. Downstream manual curation of genes (see below) was applied to filter away any transcript chimeras. The same procedure was performed on each of the four owl sequencing libraries. The transcriptome sequencing data have been deposited in the NCBI SRA database under the following BioProject ID: PRJNA497817.

2.6. Gene sequences and gene identification

To identify transcripts of genes involved in phototransduction or the synthesis of oil droplet carotenoids a similarity-based approach was used. A bird gene database was built with protein sequences for all coding regions from Peregrine falcon (*Falco peregrinus*) RefSeq Accession PRJNA198010, Barn owl (*Tyto alba*) RefSeq Accession PRJNA263629, Bald eagle (*Haliaeetus leucocephalus*) RefSeq Accession PRJNA269279, Zebra finch (*Taenopygia guttata*) RefSeq sequences TaxID 59,729 and chicken (*Gallus gallus*) RefSeq Accession PRJNA10808, which were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov). The bird gene database was then used as query sequences to blast against each owl transcriptome assembly using blastx 2.6.0+ (Zhang, Schwartz, Wagner, & Miller, 2000) with an e-value cut-off of 1e-10. All assembled genes were also mapped against a chicken genome (GenBank accession AADN00000000.4) with the bwa v0.7.15 software, using the bwa-mem algorithm (Li, 2013). All transcripts that had a blast hit among the genes involved in the phototransduction pathway in rods and cones (according to KEGG pathway: map04744 and Lamb, 2013, Figure 26) and the four selected enzymes belonging to the carotenoid biosynthesis pathways (KEGG pathway: map00906) were then extracted. Each transcript was annotated according to its best blastx hit. If more than one transcript was annotated to the same gene of interest the longest transcript was kept for further analysis. Alternatively, if two fragments from the same gene overlapped, they were manually assembled to full length genes. This assembly and assignment method was verified by checking that the fragments of the assembled genes of interest were mapped to the correct gene location in the chicken genome and that assembled genes were correctly aligned to their respective chicken gene homolog. Six additional trinity assembled owl retinal transcriptomes (Long-eared owl (*Asio otus*), Little owl (*Athene noctua*), Eurasian eagle-owl (*Bubo bubo*), Indian scops owl (*Otus bakkamoena*), Eurasian scops owl (*Otus scops*) and Eastern grass owl (*Tyto longimembris*)) retrieved from Wu et al. (2016) were also used in the analysis and treated with the same procedure.

2.7. Opsin gene trees

Amino acid sequences for the owl cone (LWS, SWS2, RH2) and rod (RH1) opsins were extracted and aligned in Ugene v. 1.26.0 (Golosova et al., 2014) with the Clustal Omega algorithm (Sievers et al., 2014) and five iterations. To generate separate gene trees of each photopigment gene, the longest continuous gene stretches without any gaps in all nine owl species were used (312aa for LWS, 178aa for RH2, 162aa for SWS2 and 191aa for RH1). Gaps were allowed when constructing a combined gene tree for all pigment sequences. Bootstrap consensus trees with 500 replicates were constructed in RaxML (Stamatakis, 2014) with the JTT model. The output data files were imported in R v.3.3.3 (R Development Core Team) with the phytools package (Revell, 2012) for output visualization.

2.8. Selection analysis of phototransduction genes and carotenoid synthesis genes

Sequence alignments for genes in the phototransduction and carotenoid pathways were performed in the same manner as for the opsin genes. The longest alignment without any gaps from each gene was used for selection analysis. Not all genes were found in all species, and only genes with $\geq$50aa alignment without gaps from at least four species were used for further analysis. Positively selected sites in the phototransduction genes were estimated with CODEML in the PAML 4.9 package (Yang, 2007; Chang et al., 2012) under the random site models of nearly neutral (M1), positive selection (M2), β (M7) and $\beta + \omega$ (M8). Model 2 and 8 (positive selection) were tested against model 1 and 7 (neutral) respectively, using integrated Bayesian statistics in CODEML (Yang, 2006).

The number of non-synonymous substitutions per non-synonymous site (dN) and synonymous substitutions per synonymous site (dS) were estimated in codeml (Yang, 2007; Chang et al., 2012) under the M0 model. Additionally, dN and dS were estimated in MEGA7 (Kumar, Stecher, & Tamura, 2016) based on the overall mean. The Nei-Gojobori method was used (Nei & Gojobori, 1986) and standard errors were estimated by bootstrapping with 500 replicates. The differences between dS and dN were determined using Z-tests of selection.

2.9. Abundance estimation ratios of genes in the phototransduction and carotenoid synthesis pathways

Transcript quantification was done with Perl scripts in the Trinity toolkit (Grabherr et al., 2011). The abundance estimation was performed with the Perl script align_and_estimate_abundance.pl. It produces transcript-level estimates of the count of fragments that were derived from each transcript. The RSEM (RNA-seq by Expectation-Maximization) was used to produce the expression values. The abundance_estimates_to_matrix.pl script was then used to generate gene and isoform expression matrices. The gene matrix was used for downstream

analysis. The RSEM expression values were checked for trinity transcripts that had a blast hit within the vision genes. If the same vision gene was represented by several Trinity transcripts, the RSEM values were added up.

The expression ratios of each opsin gene were visualized with R, for the overall opsin transcript ratios and the cone-specific opsin transcript ratios. The expression values for the other genes involved in phototransduction were visualised separately. The R/Bioconductor package pathview, a tool set for pathway based data integration and visualization (Luo & Brouwer, 2013), was used to render the graphs. The KEGG map 04744 (phototransduction) was used with chicken (species = gga) as reference organism.

2.10. Ocular transmittance

Ocular media transmittance was measured as described in Lind, Mitkus, Olsson and Kelber et al. (2013, 2014). In short, the eye was enucleated, and on the posterior pole, a circular piece of sclera, choroid and retina was removed, leaving the vitreous humour as intact as possible. The eye was rinsed with 340 mOsmol kg^{-1} phosphate buffered saline (PBS) and placed with the posterior pole up in a black plastic container filled with PBS with a circular (5 mm) fused silica window in the bottom. Light from a PX2-Xenon lamp (Ocean Optics) illuminated the cornea via a light guide through the fused silica window, and the transmitted light was collected by a light guide connected to a spectroradiometer (Maya, Ocean Optics) controlled by Spectrasuit software (v. 1.0, Ocean Optics). The transmittance of the container filled with PBS-solution was measured as reference. The measurements were smoothed by an 11-point running average and normalized to the highest value within the range of 300–700 nm. From these curves, the lowest wavelength, at which 50% of the light incident on the cornea was transmitted to the retina ($\lambda_{T0.5}$) was determined. For species, for which we already had data (Supplementary data in Lind et al., 2014), we combined these with the newly obtained results and calculated a species average. For body sizes, we consulted the bird weight data compiled by Dunning (2007).

3. Results

3.1. PCR amplification of SWS1

Using SWS1 specific primers that had been tested in many species of birds before, we attempted to amplify SWS1 sequences from the Tawny owl (*Strix aluco*) and Boreal owl (*Aegolius funereus*). No SWS1 transcripts were successfully amplified from either of the two retina samples from Tawny owl and Boreal owl despite successful amplification of the SWS1 gene with these primers in both New World and Old World warblers by Bloch et al. (2015) as well as many other divergent species of passerine and non-passerine birds (NIB, unpublished data).

3.2. Owl retinal transcriptomes

The sequencing of the four retinal transcriptomes generated a total of 6,234,082 paired reads from Long-eared owl, 7,352,305 from Short-eared owl, 5,409,204 from Tawny owl and 6,486,664 from Boreal owl. After trimming 5,592,357, 6,600,808, 4,788,946 and 5,808,313 paired reads were left for the four species, respectively. The number of transcripts in the Trinity assembly was 278,585 for Long-eared owl, of which 69,664 (25%) had a blastx hit to the custom-made bird gene database, 342,205 for Short-eared owl of which 74,935 (22%) had a blastx hit, 223,498 for Tawny owl, of which 63,030 (28%) had a blastx hit and 312,908 for Boreal owl of which 72,197 (23%) had a blastx hit.

3.3. Short wavelength-sensitive cone opsin 1 (SWS1)

The *de novo* assembled genes were blasted against the custom-made bird gene database, and there was no match for the SWS1 gene in any of the examined owls. When mapped against the reference genome of chicken, no reads or transcripts mapped to the scaffold with the SWS1-gene locus, although roughly 95% of all reads and transcripts mapped successfully onto the reference genome. Based on these findings it is very likely that the UV/V cone pigment is not expressed at any in these owl species.

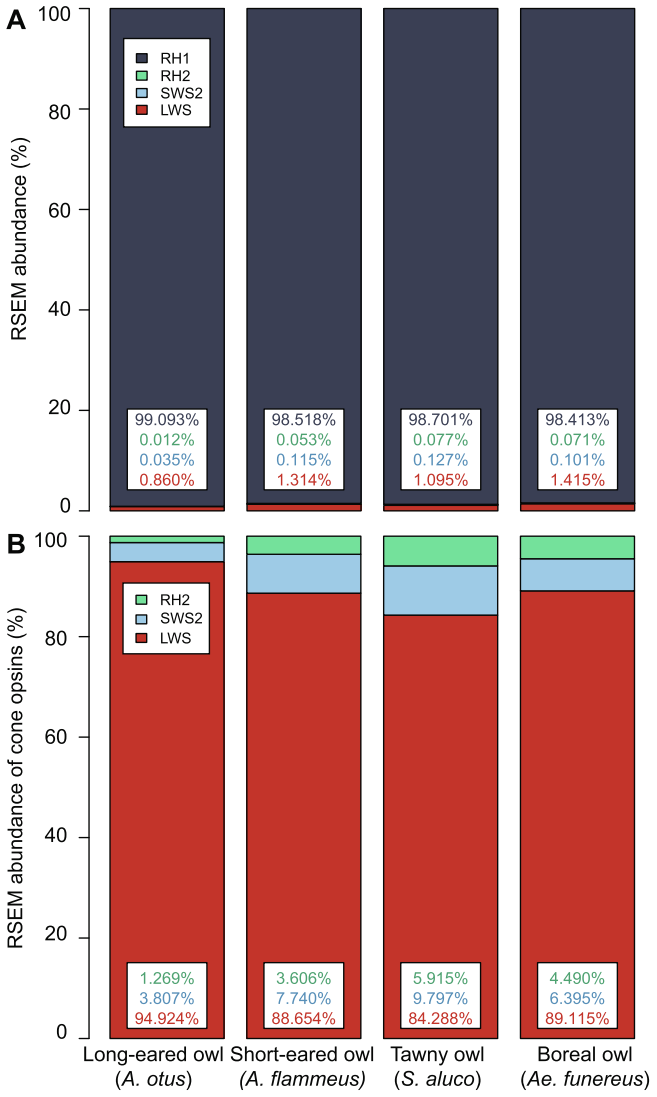

Fig. 1. Transcript abundance of opsin genes in owl retinae. (A) Relative abundance of rod opsin and cone opsin transcripts from four owl retinal transcriptomes. (B) Relative abundance of cone opsin transcripts from the four transcriptomes. Abundance was estimated with the RSEM method.

3.4. Opsin gene expression ratios

The relative expression of all opsin genes (LWS, RH1, RH2 and SWS2) out of all retinal transcripts in the four species was 3.3% for Long-eared owl, 3.3% for Short-eared owl, 3.6% for Tawny owl and 4.5% for Boreal owl. As expected, the rhodopsin RH1 had the highest relative expression of the four opsin genes, accounting for 98–99% of the abundance in all four species (Fig. 1A). Among the cone opsins (Fig. 1B), LWS had the highest expression, followed by SWS2 and then RH2.

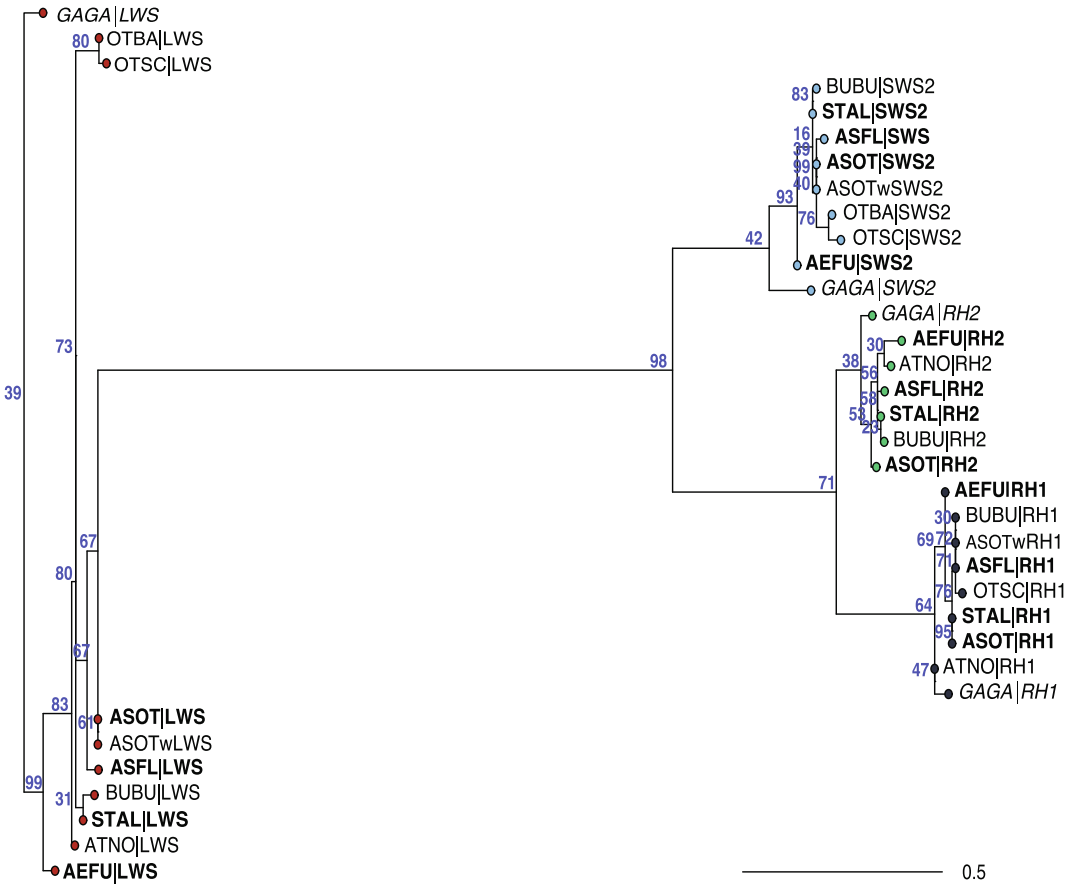

Fig. 2. Maximum likelihood tree of owl and chicken opsin genes (LWS, RH1, RH2 and SWS2) based on amino acids and produced in RaxML under the JTT model of sequence evolution. Chicken, *G. gallus* (GAGA) LWS was used as outgroup. Bootstrap values are shown in blue at each node. Bold text indicates sequences obtained in this study, normal font those from Wu et al. (2016), and italic the outgroup. The owl species are: AEFU – *Aegolius funereus,* ASFL – *Asio flammeus,* ASOT – *Asio otus,* ATNO – *Athene noctua,* BUBU – *Bubo bubo,* OTBA – *Otus bakkamoena,* OTSC – *Otus scops,* STAL – *Strix aluco,* TYLO – *Tyto longimembris.* The *A. otus* specimen from this study are marked as ASOT|, those from Wu et al. (2016) are marked as ASOTw. Scale: substitutions per site.

3.5. Opsin gene trees

Each opsin gene transcript (LWS, RH1, RH2 and SWS2) from nine owls clusters separately in a combined Maximum Likelihood tree (Fig. 2). The RH1 and RH2 clusters have fairly high support (71%), and the SWS2 cluster has very high support (93%). The LWS gene differs the most and the branching between LWS and the other opsin genes has high support (98%). Within each cluster (i.e. each opsin gene) there is little differentiation (i.e. short branch length) between the species, although the branch length for chicken SWS2 is slightly longer than for RH2 and RH1. For all opsin genes, chicken (GAGA) lies as a sister group to all the owls but with quite low support (30–50%). The highest support in all clusters is found for closely related species – i.e. those belonging to the same genera. In the separate gene trees, most support values are between 60 and 80% (Fig. 3), and branch lengths are short. All species are from the family of Strigidae except the Eastern grass owl, which belongs to Tytonidae. When the Eastern grass owl sequences could be used in the alignments (LWS and RH1), sequences clustered with those of Strigidae.

3.6. Selection analysis

The non-synonymous (dN) to synonymous (dS) substitution rate differs significantly from one for 18 out of 25 genes in the phototransduction pathway (Fig. 4; Table 1; Z-test of selection, $p < 0.05$), and ω (dN-dS ratio) is lower than one, which means that these genes are overall under purifying selection. Four genes, cyclic nucleotide gated channel beta 3 (CNGB3), S-antigen visual arrestin (SAG), SWS2 and LWS, do not show a significant difference ($p < 0.05$) of synonymous and non-synonymous mutation rates. The remaining genes (CALM, PDE6, PDE6B) had no non-synonymous substitutions in this dataset (Table 1).

Even though most genes in the phototransduction pathway are overall under purifying selection, four genes had signatures of positive selection at some sites ($\chi 2$: $p < 0.05$: $2\Delta lnL > 5.9915$, $df = 2$): cyclic nucleotide gated channel beta 1 (CNGB1/CNG), G protein subunit beta 1 (GNB1/Gt), phosphodiesterase 6B (PDE6B/PDE), and SAG/Arr (Supplementary Table S1).

3.7. Phototransduction gene expression

Rod-specific pathway. Rhodopsin was by far the most highly expressed gene in the entire pathway (Supplementary Fig. S1) in all four species. The genes transducin (GNAT1/2/GNB1/Gt) and guanylate cyclase (GUCY2E/F) also showed very high expression levels in the retina of the owls.

Cone-specific pathway. Generally, arrestin (Arr) was the most expressed gene, followed by regulator of protein signaling 9 (RGS9) and the guanylate cyclases (GC) (Supplementary Fig. S2). The Short-eared owl had a generally higher expression of recoverin (RCVRN/Rec) and transducin (Gt) than the other three species.

3.8. Oil droplet carotenoid synthesis genes

The *de novo* assembled genes were blasted against the custom-made bird gene database, and to the CYP2J19 gene. Within the carotenoid

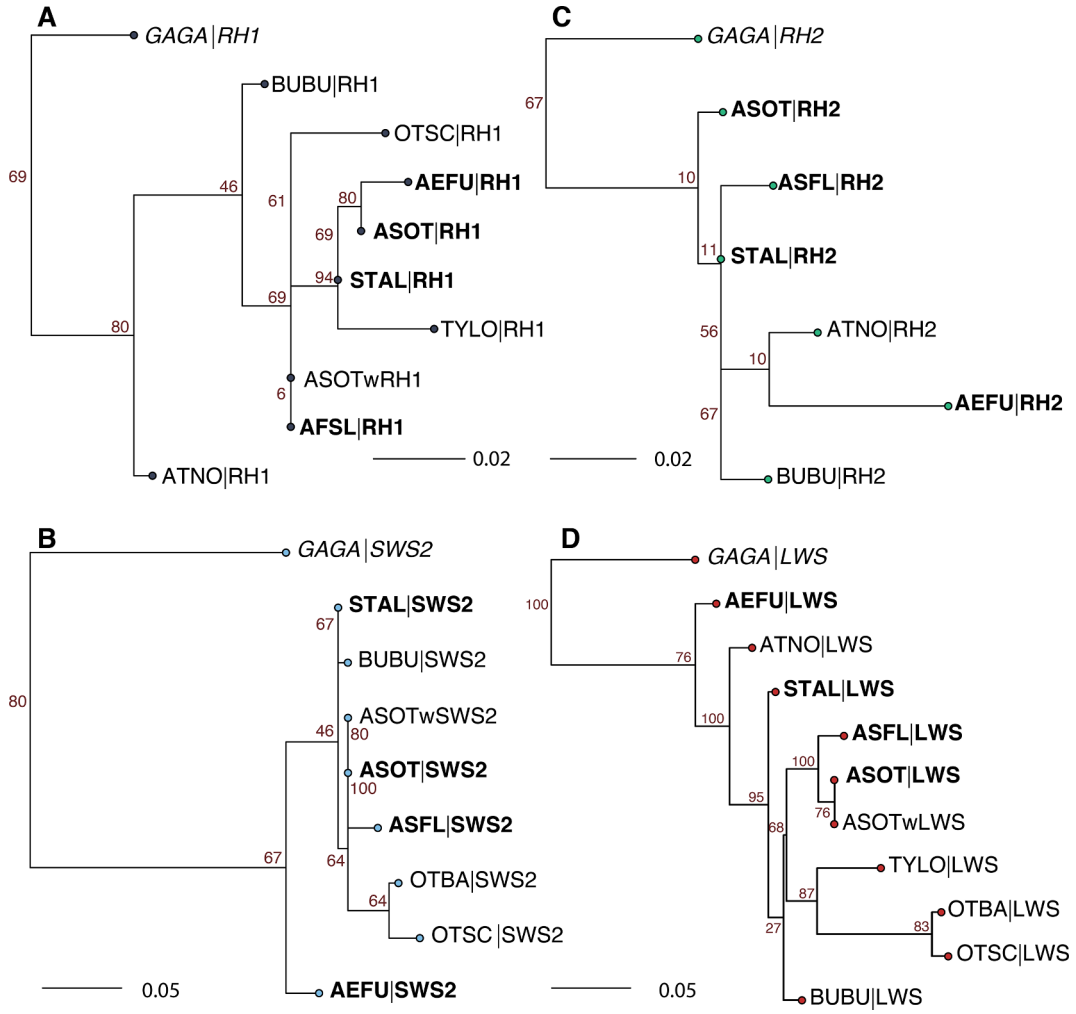

Fig. 3. Separate maximum likelihood trees of the opsin genes in owls produced in RaxML under the JTT model of sequence evolution. Chicken opsins (GAGA) were used as outgroup. Bootstrap values are shown in red at each node. (A) Rod opsin RH1. (B) SWS2, (C) RH2, (D) LWS. Bold text indicates sequences obtained in this study, normal font those from Wu et al. (2016), and italic font the outgroup. Scale: substitutions per site. Species are abbreviated as in Fig. 2.

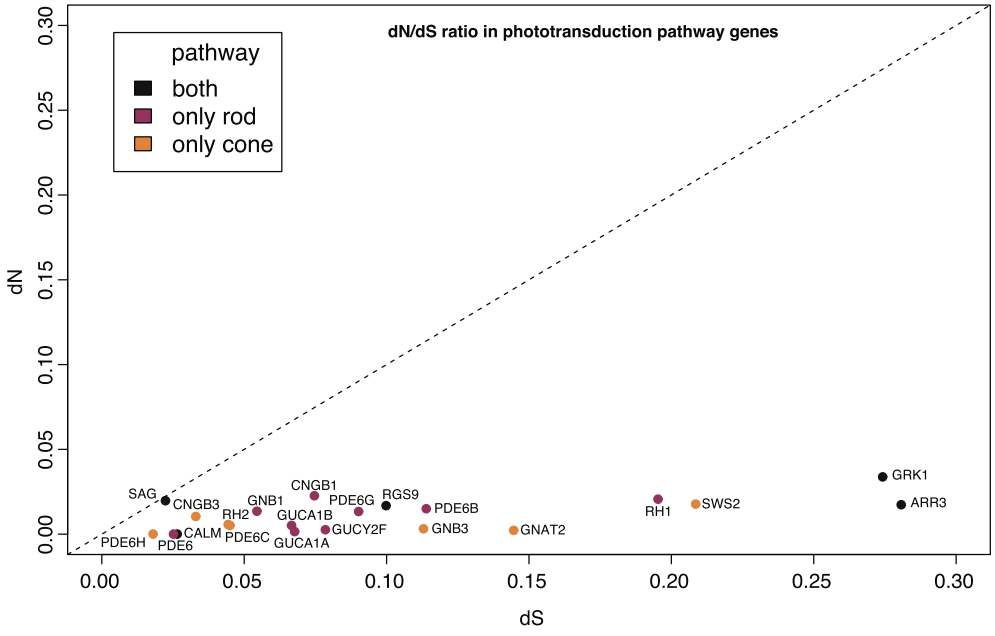

Fig. 4. Average evolutionary divergence in genes involved in the phototransduction pathway in nine owl species (see text for names). Non-synonymous (d_N) to synonymous (d_S) rates. Genes below the line have lower non-synonymous than synonymous substitution rates. Analyzes were conducted using CODEML in the PAML 4.9 package (Yang, 2007; Chang et al., 2012). The genes are: retinal cone arrestin (ARR3), calmodulin (CALM), cyclic nucleotide gated channel beta 1 (CNGB1), cyclic nucleotide gated channel beta 3 (CNGB3), G protein subunit alpha transducing 1 (GNAT1), G protein subunit alpha transducing 2 (GNAT2), G protein subunit beta 1 (GNB1), G protein subunit beta 3 (GNB3), G protein-coupled receptor kinase 1/7 (GRK1/7), guanylate cyclase activator 1A (GUCA1A), guanylate cyclase activator 1B (GUCA1B), guanylate cyclase 2F (GUCY2F), phosphodiesterase 6 (PDE6), phosphodiesterase 6B (PDE6B), phosphodiesterase 6C (PDE6C), phosphodiesterase 6G (PDE6G), phosphodiesterase 6H (PDE6H), recoverin (RCVRN), regulator of G-protein signaling 9 (RGS9), S-arrestin (SAG), rhodopsin (RH1), medium wavelength-sensitive opsin (RH1), short wavelength- sensitive opsin (SWS2), long wavelength-sensitive opsin (LWS).

Table 1
Codon-based evolutionary divergence of genes involved in the phototransduction pathway in nine owl species. N = the number of owl species from which the specific gene (or gene fragment) could be retrieved from retinal transcriptomes. The values for dN, dS and dN/dS were estimated in CODEML under the neutral model, M0.

Gene	N	Length[i] (bp)	d_N[ii]	d_S[iii]	d_N/d_S
ARR3	10	1071	0.017	0.281	0.062
CALM	9	432	0.000	0.027	0.000
CNGB1	9	1317	0.023	0.075	0.304
CNGB3	6	213	0.010	0.033	0.316
GNAT1	7	711	0.039	0.748	0.052
GNAT2	6	189	0.002	0.145	0.016
GNB1	8	1017	0.014	0.055	0.248
GNB3	6	1020	0.003	0.113	0.027
GRK1	8	1425	0.034	0.274	0.123
GUCA1A	10	282	0.002	0.068	0.024
GUCA1B	10	465	0.005	0.067	0.078
GUCY2F	6	585	0.003	0.079	0.033
PDE6	7	450	0.000	0.025	0.000
PDE6B	9	1968	0.015	0.114	0.132
PDE6C	9	1713	0.005	0.045	0.113
PDE6G	8	261	0.013	0.090	0.148
PDE6H	6	168	0.000	0.018	0.000
RCVRN	10	561	0.026	0.787	0.033
RGS9	10	630	0.017	0.100	0.169
SAG	7	330	0.020	0.022	0.888
RH1	9	573	0.021	0.195	0.106
RH2	4	537	0.006	0.044	0.129
SWS2	8	450	0.018	0.209	0.085
LWS	10	939	0.058	0.648	0.090

[i] Total length in base pairs of analyzed gene segment.
[ii] Non-synonymous substitution rate as defined by the tree length for dN under the M0 model.
[iii] Synonymous substitution rate as defined by the tree length for dS under the M0 model.

biosynthesis pathway, there was no match in any of the examined owls. When mapped to the reference genome of chicken, only transcripts with a best hit within the cytochrome p450 enzyme CYP2J2 mapped against the CYP2J19 region of chicken. CYP2J2 does not have a function within the formation of ketocarotenoids, it is involved in synthesis of cholesterol, steroids and other lipids (Refseq 2016 Information retrieved at National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov at 2018-05-10) it likely mapped onto the CYP2J19 region due to sequence similarity in certain parts of the gene, as expected for proteins of the same family. CYP2J19 is not expressed at all in these owl species or alternatively, is expressed at very low levels and thus not detected. Based on this, these owl species might not be able to synthesize the red ketocarotenoid astaxanthin present in the L cone oil droplets in retinae of other birds.

By contrast, transcripts of enzymes required to produce the apocarotenoids galloxanthin and dihydrogalloxanthin from zeaxanthin, beta-carotene oxygenase 2 (BCO2), retinol dehydrogenase 12 (RDH12), and retinol saturase (RETSAT) were found in all owl species (Fig. 5) at different abundance levels. The fact that RETSAT - required to produce dihydrogalloxanthin from galloxanthin – was the least-expressed gene in all species may indicate that both apocarotenoids are likely present in the S cone oil droplets of owls (see Toomey et al., 2016).

3.9. Ocular transmittance

The lowest wavelength, at which the ocular media transmitted 50% of the incoming light, $\lambda_{T0.5}$, was 350 nm for the eye of the Short-eared owl, 359 nm for the Eurasian eagle owl, 341 nm for the Boreal owl, 344 nm for the Long-eared owl, and 345 and 365 nm for the two Tawny owl eyes. These values are similar to those reported for other owls (Lind et al., 2014). Fig. 6 shows the averaged ocular media transmittance curves for all owl species, for which data are available: Short-eared owl,

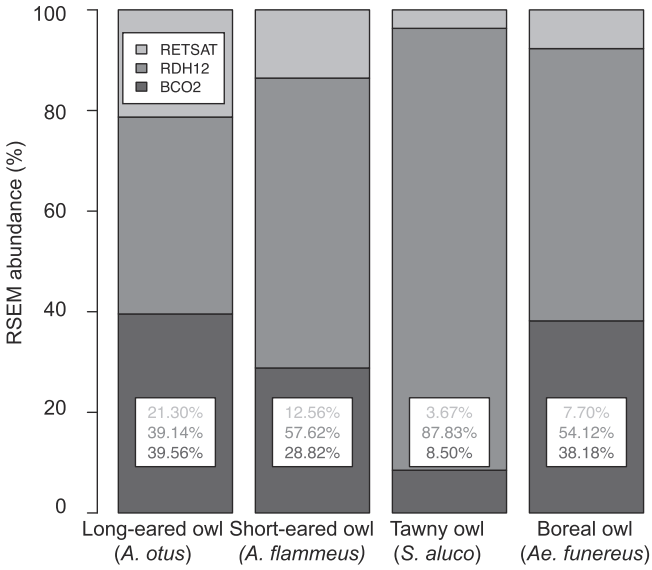

Fig. 5. Transcript abundance of enzymes in the galloxantin pathway in owl retinae. Relative abundance of beta-carotene oxygenase 2 (BCO2), retinol dehydrogenase 12 (RDH12), and retinol saturase (RETSAT) transcripts from four owl retinal transcriptomes. Abundance was estimated with the RSEM method.

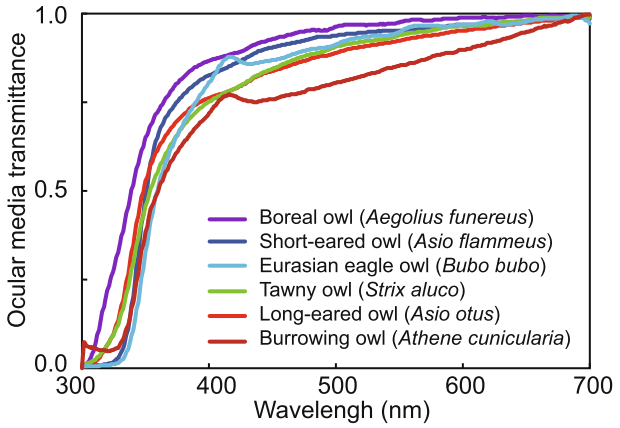

Fig. 6. Ocular media transmittance of six species of owls. See text for details and Supplementary data file Höglund_et_al_2018_Owl_OMT for full spectral transmittance data.

Eurasian eagle owl, Long-eared owl (N = 2 animals; n = 2 eyes; $\lambda_{T0.5}$ 348 nm), Tawny owl (N = 3; n = 4; $\lambda_{T0.5}$ 346 nm), Boreal owl (N = 2; n = 2; $\lambda_{T0.5}$ 339 nm) and Burrowing owl (*Athene cunicularia*; N = 1; n = 2; $\lambda_{T0.5}$ 360 nm; see Supplementary data file Höglund_et_al_2018_Owl_OMT.xlsx for the data). Inter-species differences are small but the ocular media of the largest species, the Eurasian eagle owl (weight 2686 g; Dunning, 2007), has a long-wavelength-shifted $\lambda_{T0.5}$, and thus relatively low UV transmittance, compared to most other, smaller species. The exception is the Burrowing owl, which is the most diurnally active species in our sample. Although one of the smallest species (body weight 151 g; Dunning, 2007), it has almost the same $\lambda_{T0.5}$ as the Eurasian eagle owl.

4. Discussion

Our analysis of retinal transcriptomes of four owl species has confirmed the absence of transcripts of the ultraviolet-/violet-sensitive cone opsin SWS1, whereas the green-sensitive cone opsin RH2 had a low expression level in all examined species. Overall, genes important for the phototransduction pathway were under purifying selection, but

several genes, mostly in the rod-specific pathway, had positively selected sites. The rod opsin RH1 was the opsin with the highest expression, confirming earlier results that the owl retina is rod-dominated. Since the SWS1 opsin has not been found in any owl species investigated so far, these findings add further support for the hypothesis that owls have lost the SWS1 opsin. Despite their lack of an SWS1 pigment, all owls showed high transmittance of the ocular media for UV light, compared to diurnal raptors. We interpret the findings as adaptations to the nocturnal lifestyle of owls.

4.1. Rod-dominated retinae in nocturnal vertebrates

Most nocturnal vertebrates, including almost all mammals (see Kelber, 2018; but see Kryger, Galli-Resta, Jacobs, & Reese, 1998) have rod-dominated retinae, whereas most birds have cone-dominated retinae (Hart, 2001b) and the retinal area with highest resolution, the area centralis or fovea, tends to be rod-free (e.g. Coimbra, Collin, & Hart, 2015; Mitkus, Olsson, Toomey, Corbo, & Kelber, 2017). Owls, by contrast, have a retina that mainly contains rods (Walls, 1942; Martin, 1990), even in the fovea (Fite, 1973; Oehme, 1961).

This is reflected by our finding that 98–99% of the opsin transcripts in all studied owl retinae belong to RH1, that is expressed in rods (Fig. 1). No clear differences were detected between species, but since the time of death was not the same for all specimens, and only one replicate was available per species, the results cannot be statistically compared.

4.2. The loss of SWS1 opsins in nocturnal vertebrates

In the retinae of most birds, between 5 and 10% of all cones are UV/V cones expressing SWS1 opsin (Hart, 2001a). We did not find SWS1 transcripts any of the four owl species using PCR amplification and transcriptome sequencing, which is in agreement with previous studies using microspectrophotometry (Bowmaker & Martin, 1978), genomics (Borges et al., 2015; Hanna et al., 2017) and transcriptomics (Wu et al., 2016). Our results further support the hypothesis that owls have lost a functional SWS1 gene early in evolution.

Interestingly, while Strigiformes have lost tetrachromatic vision, another mostly nocturnal clade of birds, the Caprimulgiformes (nightjars and allies), seem to have kept a functional SWS1 opsin (Ödeen & Håstad, 2003). Similarly, in some nocturnal mammals, including several nocturnal primates, carnivores and bats, the SWS1 opsin gene has also undergone pseudogenization (Jacobs, 2013). However, not all mammals that lack the SWS1 gene have a nocturnal lifestyle, and not all nocturnal mammals have undergone SWS1 gene pseudogenization (Jacobs, 2013).

4.3. Expression patterns of the remaining cone opsin pigments

In all four species of owls studied here, transcripts of the LWS opsin gene made up 85–95% of all cone opsin transcripts (Fig. 1B). In bird retinae, LWS opsin is expressed in L single cones and in D cones (Hart, 2001b). In the chicken (Bowmaker & Knowles, 1977; Kram, Mantey, & Corbo, 2010) and over 20 other bird species from 11 orders (Hart, Partridge, & Cuthill, 1998; Hart, 2001a), 50–70% of cones express LWS opsin ($\approx 20-50\%$ D cones plus $\approx 10-20\%$ L cones), $\approx 15-20\%$ are M cones expressing RH2 and $\approx 5-20\%$ are S cones expressing SWS2. The high expression levels of LWS opsin may indicate an unusually high abundance of D cones in the studied owl retinae. In retina samples from the Great horned owl and the Barred owl (*Strix varia*), D cones made up 67% and 75% of all cones, respectively (Braekevelt (1993); Braekevelt, Smith, & Smith, 1996), but it is unknown, from which retinal region these samples were taken. In our samples, SWS2 opsin was expressed about twice as much as RH2 opsin.

However, cone ratios can differ across the dorso-ventral and the naso-temporal axes of a bird retina (Hart et al., 1998; Hart, 2001a), which can affect the transcript abundances we have observed, as undetermined retinal quartiles were examined. Moreover, the owls were not euthanized at the same time of the day, and opsin gene expression ratios may underlie a circadian rhythm (Korenbrot & Fernald, 1989; Pierce et al., 1993). The relevance of the observed cone opsin expression levels for spectral sensitivity and colour vision of owls remains to be studied with behavioural methods.

4.4. Molecular evolution and expression patterns in the phototransduction pathway

None of the studied genes had an overall higher non-synonymous (dN) than synonymous (dS) substitution rate (Fig. 4 and Supplementary Table S1. A dN/dS value above 1 shows that the gene is under positive selection whereas a value below 1 indicates purifying selection. The dN and dS values are computed as overall averages for the whole protein sequence, and if a few specific sites are under positive selection (such as spectral-tuning sites in opsin genes) while the remainder of the gene is under purifying selection, the positively selected sites will not strongly influence the gene selection average. Overall, the studied genes had low non-synonymous substitution rates, showing that their amino acid sequences are largely conserved among the investigated species. All genes with positively selected sites (Supplementary Table S1) show dN/dS-ratios below 1, indicative of genes under purifying selection.

Positively selected sites were found in rod-specific isoforms of genes within the phototransduction pathway, and in genes that rod and cone pathways have in common (Supplementary Table S1): G protein subunit beta 1 (GNB1), cyclic nucleotide gated channel beta 1 (CNGB1), S-antigen visual arrestin (SAG) and phosphodiesterase 6b (PDE6B). PDE6B and CNGB1 are involved in the activation of phototransduction and SAG is involved in the photo response recovery or adaptation (Wu et al., 2016). GNB1, the rod-specific beta-unit of transducing, is involved in light-adaptation. It activates the phosphodiesterase cascade (the PDE6 isoforms) during phototransduction. Wu et al. (2016) found the same genes to have positively selected sites in the owl species that they studied (except GNB1). They also found positive selection in solute carrier family 24 member 1 (SLC24A1, which was too fragmented to be analysed in the present study), LWS, SWS2, cyclic nucleotide gated channel alpha 1 (CNGA1), protocadherin related 15 (PCDH15) and ATP binding cassette subfamily A member 4 (ABCA4) (the latter two genes were not included in the present study). Wu et al. (2016) argued that the selection found in “dim light genes” with functions in activation and recovery of photoreceptors may contribute to enhanced absolute sensitivity. Previous analysis of cone absorbance spectra of the Tawny owl indicated shifts in the absorbance peaks λ_{max} of both LWS and SWS2 opsins towards the wavelengths most dominant in twilight (450 nm), which might help to maximize photon absorption (Bowmaker & Martin, 1978; Wu et al., 2016). Even though we did not find significantly positively selected sites in these genes, but they were the most expressed cone opsins in all four species (Fig. 2). Bloch (2016) also found differences in opsin expression that are relevant to avian species ecology, suggesting variation in opsin expression could be a useful mechanism to adapt visual systems for special light environments.

The genes in both the rod- and cone-specific phototransduction pathway that were most strongly expressed (after RH1) in the four owl retinal transcriptomes were SAG (Arr), guanylate cyclases (GC) and RGS9 (Supplementary Figs. S1–S3). Guanylate cyclase is involved in the cGMP synthesis and SAG in photoresponse recovery and adaptation. The higher expression of SAG may lead to increased visual sensitivity and the positively selected sites found in this gene might reflect differences in selection pressure on photoresponse recovery and adaptation among the four examined owl species. Interestingly, the rod-specific isoforms of transducin (GNB1, Gt in Supplementary Fig. S3) was highly expressed in two of the owl species, Short-eared owl and Boreal owl, compared to the cone-specific isoforms and the other genes. GNB1 was also found to have positively selected sites. It plays a role in the

activation of phototransduction and the response is directly proportional to the amount of transducin activated, which then activates PDE (Lodish, Berk, & Zipursky, 2000).

As expected, rhodopsin was the gene with highest expression in the rod-specific phototransduction pathway (Supplementary Fig. S2), followed by transducin (GNB1/GNB3). The fact that GNB1 is also acted upon by selection (together with PDE6B) might indicate optimization of the phototransduction cascade activation under dim light conditions. In future studies, it would be valuable to perform a differential expression analysis to see whether the expression of genes in phototransduction cascade differs between owls with different activity patterns.

4.5. Genes in the carotenoid pathways

All enzymes required to produce the apocarotenoids galloxanthin and dihydrogalloxanthin, were expressed in the owl retinae (Fig. 5). Although we did not measure pigment density, their S cone oil droplets likely have similar pigmentation to those of other birds, and the resulting spectral sensitivity of S cones should thus be similar to those of other birds, most likely to those with V cones. By contrast, due to the loss of CYP2J19, L cone oil droplets are not densely pigmented with astaxanthin. In the Tawny owl, Bowmaker and Martin (1978) found L cones with visual pigment maximally absorbing at 555 nm and pale yellow oil droplets, but no intact cones with red oil droplets. The most parsimonious scenario thus is that L cone oil droplets of owls are similar to M cone and D cone oil droplets, as has been observed in canary birds (Das, Wilkie, Hunt, & Bowmaker, 1999), and the spectral sensitivity of the L cone is much broader than in other birds (Fig. 7). This, in turn, would help to capture more photons in mesopic light levels when both rods and cones are active. Similar to owls, kiwis and penguins have recently been found to have lost a functional gene for CYP2J19 (Emerling, 2018).

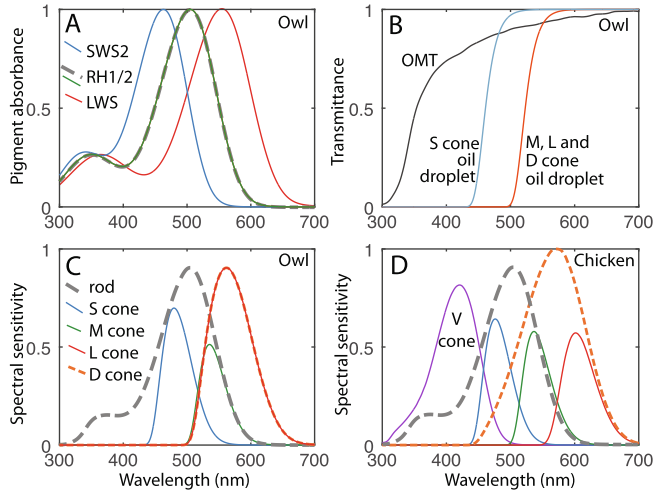

Fig. 7. Photoreceptor sensitivities of the Tawny owl, compared to the chicken. A. Normalized spectral absorbance of rod (RH1) and cone (SWS2, RH2, LWS) visual pigments, B. Ocular media transmittance (OMT) and cone oil droplets of short-, medium- and long wavelength-sensitive (S, M and L) single cones and double (D) cones, C Expected spectral sensitivity of the rods and cones of the Tawny owl. Note that the sensitivity is reduced due to the filter effects, and that double cones likely have the same sensitivity as the L cone. D. Chicken photoreceptors, including the violet-sensitive (V) cones, which are likely absent in the owl. The other sensitivity curves are coded as in C. L cones have much lower sensitivity as they have a different type of oil droplet in the chicken. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.6. High ocular media transmittance for UV light – An adaptation to highly sensitive rod vision

The transmittance of the ocular media for ultraviolet limits the amount of UV light that reaches the retina. UV light is assumed to damage retinal tissue, thus animals that do not see UV should limit UV-transmittance. Accordingly, the wavelength, at which the ocular media transmit 50% of the incoming light, $\lambda_{T0.5}$, is correlated with the peak absorbance of the SWS1 opsin (Lind et al., 2014). Specifically, $\lambda_{T0.5}$ of diurnal raptors has been found to be high, resulting in very low sensitivity of these birds to UV light.

The surprisingly low $\lambda_{T0.5}$ of owls, which have lost SWS1 gene expression completely, can only be explained as an adaptation to increase the absolute sensitivity of their rod-based vision in dim light. The spectral composition of light is strongly short-wavelength-shifted, with an intensity peak around 450 nm, during the hours between sunset and the end of astronomical twilight, when the sun is 18° below the horizon and no longer contributes to illumination (Johnsen et al., 2006). Crepuscular species strongly benefit from enhanced UV sensitivity. Under these conditions, the UV-reflecting white patches of conspecifics will appear slightly brighter to the owls due to the UV-sensitivity of their rods. Interestingly, the most diurnal species in our sample, the Burrowing owl, which may benefit the least from UV light, had the $\lambda_{T0.5}$ at the highest wavelength (360 nm).

4.7. Putative spectral sensitivity of the Tawny owl

Combining our results on the absence of SWS1 opsin and CYP2J19 transcripts in owl retinae and the ocular media transmittance of their eyes with the MSP data reported by Bowmaker and Martin (1978), we can deduce the likely spectral sensitivity of the photoreceptors of the Tawny owl (Fig. 7). We assume that the L cone oil droplet is pigmented with zeaxanthin. Compared to the chicken (Fig. 7D) the owl (Fig. 7C) has a much narrower spectrum (see also Wu et al., 2016), but higher absolute sensitivity of cone vision. Behavioural tests of owl spectral sensitivity are needed to confirm these results and to further increase our understanding of the visual adaptations of owls. The only two behavioural studies on owl colour vision showed that Little owls (Meyknecht, 1941) and Tawny owls (Martin, 1974) can discriminate colours under bright light conditions.

Author contributions

AK, MM, PO and OL conceived the project. PO and MM collected eyes, extracted retinae and performed ocular transmittance measurements. PO and AD processed the RNA. JH analysed transcriptomes under the supervision of MS. PO and AD did reverse transcription experiments, with support from NIB. JH, AK and MS wrote the paper with contributions from all other authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.visres.2019.02.005.

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