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LWS visual pigment in owls: Spectral tuning inferred by genetics

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ABSTRACT

Owls constitute a diverse group of raptors, active at different times of the day with distinct light conditions that might be associated with multiple visual adaptations. We investigated whether shifts in the spectral sensitivity of the L cone visual pigment, as inferred by analysis of gene structure, could be one such adaptive mechanism. Using Sanger sequencing approach, we characterized the long wavelength-sensitive (LWS) opsin gene expressed in the retina of five owl species, specifically chosen to represent distinct patterns of activity. Nocturnality was epitomized by the American barn owl (*Tyto furcata*), the striped owl (*Asio clamator*), and the tropical screech owl (*Megascops choliba*); diurnality, by the ferruginous pygmy owl (*Glaucudium brasilianum*); and cathemerality, by the burrowing owl (*Athene cunicularia*). We also analyzed the presence of the L cone in the retinas of four species of owl (*T. furcata, A. cunicularia, G. brasilianum* and *M. choliba*) using immnunohistochemistry. Five critical sites for the spectral tuning of the LWS opsin (164, 181, 261, 269, and 292) were analyzed and compared to the sequence of other birds. The sequence of *A. cunicularia* showed a substitution on residue 269, with the presence of an alanine instead threonine, which generates an estimated maximum absorption (λ_{max}) around 537 nm. No other variation was found in the spectral tuning sites of the LWS opsin, among the other species, and the λ_{max} was estimated at around 555 nm. The presence of L cones in the retinas of the four species of owls was revealed using immunohistochemistry and we observed a reduced number of L cones in *T. furcata* compared to *A. cunicularia*, *G. brasilianum* and *M. choliba*.

1. Introduction

Most bird species have diurnal activities, and only about 3% are nocturnal. Among nocturnal birds are species of owls, parrots and the kiwi (Martin, 1990, 2017). Nocturnal conditions impose important selective pressures on the sensory systems of animals, especially on vision (Martin, 1986; O'Carroll & Warrant, 2017). Optimizing visual performance under dim illumination requires optical and/or neural strategies to enhance the sensitivity of the visual system to light (Land, 1981; Land & Nilsson, 2002). In vertebrates, such adaptive strategies are often largely implemented by adjusting gross morphological features of the eye (Wood, 1917; Walls, 1942; Hall & Ross, 2006; Schmitz & Motani, 2010) and/or altering the spatial distribution and type of photoreceptors in the retina (Land & Nilsson, 2002).

Owls are one of few avian groups exhibiting a broad range of activity patterns and habitat preference. Therefore, they are an interesting group to study mechanisms of visual adaptation in birds. Owls belong to the Strigiformes order, which is further divided into two families: Tytonidae and Strigidae (del Hoyo and Elliott, 1999; König & Weick, 2008; Enríquez, Eisermann, Mikkola, & Motta-Junior, 2017, Uva, Päckert, Cibois, Fumagalli, & Roulin, 2018). Altogether, Strigiformes exhibit a number of specializations common to nocturnal vertebrates, which include large and tubular-shaped eyes to enhance retinal image brightness (Walls, 1942; Bohórquez Mahecha & Aparecida de Oliveira, 1999; Lisney, Iwaniuk, Bandet, & Wylie, 2012), and a rod-dominated retina (Fite, 1973; Martin, 1990; Oehme, 1961; Walls, 1942).

However not all owls are strictly nocturnal like the well-studied tawny owl (*Strix aluco*) (Bowmaker & Martin, 1978). Many species, such as the American barn owl (*Tyto furcata*) concentrate their activities in twilight periods, while others, such as the burrowing owl (*Athene cunicularia*), are cathemerals i.e., they have both diurnal and nocturnal activities (Martins & Egler, 1990; Motta-Junior, 2006). Other owls of

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the neotropical regions, like the stripped owl (*Asio clamator*) is active more often during crepuscular and nocturnal periods, along with some daylight activity (Motta-Junior, Alho, & Belentani, 2004). Ferruginous pygmy-owls (*Glaucidium brasilianum*) were reported as diurnal animals (Sarasola & Santillán, 2014), while the tropical screech-owl (*Megascops choliba*) is a nocturnal owl (Braga, 2006).

In the retina, two photoreceptor types, cones and rods, are responsible for capturing photons and transducing luminous information into electrical signals. These photosensitive cells have photopigments embedded in the membrane of their outer segments, which comprise an opsin protein and a carotenoid chromophore, the retinal. The opsins are G protein-coupled receptors, composed of 350–400 amino acids. When light reaches the photopigments, the 11-*cis*-retinal changes its conformation to all-trans retinal form, thereby initiating the phototransduction process (Palczewski, 2000).

Cone opsins may be identified as short-, medium- or long-wavelength sensitive on the basis of their spectral absorption peak (λ_{max}), determined by specific amino acids located at key protein locations known as spectral tuning sites. In vertebrates, four opsin genes can be expressed in different cone types, each of them codifying specific proteins with distinct spectral absorption peaks: *SWS1* (λ_{max}: 355–440 nm), *SWS2* (λ_{max}: 410–475 nm), *RH2* (λ_{max}: 460–540 nm), and *LWS/MWS* (λ_{max}: 505–630 nm). In contrast, the rhodopsin gene *RH1* is expressed in rods, with λ_{max} around 500 nm (Bowmaker, Heath, Wilkie, & Hunt, 1997; Das, Wilkie, Hunt, & Bowmaker, 1999; Hart & Hunt, 2007).

Studies about color vision and visual photopigments in owls are scarce in the literature. In two nocturnal species, *Strix aluco* and *Bubo virginianus*, the λ_{max} of the LWS opsin was measured with electrophysiological and microspectrophotometric (MSP) techniques, and both species had a spectral peak at 555 nm (Bowmarker & Martin, 1978; Jacobs, Crognale, & Fenwick, 1987). More recent studies brought important data about vision-related genes in owls, and indicated the absence of the *SWS1* opsin gene (Höglund et al., 2019), confirming previous assumptions that owls lack this gene, as it has occurred with some nocturnal animals, like crocodilian and the kiwi (Hanna et al., 2017) and also within marine e subterranean mammal species (Wu et al., 2016; Hanna et al., 2017). These studies also showed that Tytonidae owls lack *RH2* gene. Wu et al. (2016) analyzed the adaptive evolution of these genes, with one amino acid substitution in *LWS* and *SWS2* spectral tuning sites, which seem to improve photon absorption in twilight.

The *LWS* opsin gene, in birds, expresses a long-wavelength sensitive opsin with absorption peak in the range of 540–570 nm. Five amino acid residues of the LWS opsin were described to be involved in the spectral tuning of this photopigment: 164, 181, 261, 269, and 292 (Yokoyama & Radlwimmer, 1998). The presence of the amino acids serine, histidine, tyrosine, threonine and alanine (SHYTA) in these sites, usually generates a photopigment with λ_{max} at 560 nm. Variations in these amino acids may lead to shifts of the LWS λ_{max} between 543 and 571 nm in birds (Yokoyama & Radlwimmer, 1998; Bowmaker & Martin, 1985; Coyle, Hart, Carleton, & Borgia, 2012; Wright & Bowmaker, 2001).

Molecular analysis of the *LWS* gene in owls with different activity patterns is relevant to understand the evolutionary mechanisms underlying the spectral tuning of the visual opsins in birds. In this study, we analyzed the *LWS* opsin gene of five owl species, with different daily activity patterns, diurnal, nocturnal or cathemeral. We also aimed to identify the photoreceptor population containing the LWS photopigment in owls using immunohistochemistry with anti-L opsin antibody in retinal sections of the nocturnal owls *T. furcata* and *M. choliba*, the cathemeral *A. cunicularia* and the diurnal *G. brasilianum*.

2. Material and methods

2.1. Study animals

For genetic analysis, we collected the eyes of the following five owl species: burrowing owl (*Athene cunicularia*, n = 2), ferruginous pygmy owl (*Glaucidium brasilianum,* n = 1), striped owl (*Asio clamator,* n = 2), tropical screech owl (*Megascops choliba,* n = 1) and, as representative of the family Tytonidae, the American barn owl (*Tyto furcata,* n = 2). For all species except the striped owl, the eye of one individual was collected in order to compare the staining pattern of LWS.

Owls were obtained from the Wild Animal Screening Center (CETAS) of the Brazilian Institute for the Environment and Natural Renewable Resources (IBAMA) in Belo Horizonte. The animals were housed in large outdoor aviaries located close to the Institute for Biological Sciences building at the Federal University of Minas Gerais (UFMG). The permission for maintaining owls in captivity for scientific purposes was granted by IBAMA under the license n° 3076223.

All animals were euthanized by a lethal dose of sodium pentobarbital (Hypnol 3%, Fantoveter, SP). One eye of each individual was extracted and preserved in RNA stabilization solution (RNAlater™, Ambion, Massachusetts, USA) at 4 °C. The other eye was preserved in 4% paraformaldehyde (PFA) diluted in phosphate buffer saline 0.1 M (PBS) for subsequent immunohistochemical staining experiments. This study was approved by the UFMG Ethics Committee for Animal Experimentation (License n° 39/2011).

2.2. Rationale for activity pattern assignment

Owls were classified as nocturnal, diurnal and cathemeral following the rationale used by Schmitz and Motani (2010).

2.3. RNA extraction and reverse transcription

Total RNA was extracted from retinas, using the RNeasy Mini kit (Qiagen), according to manufacturer protocol. mRNA was converted to complementary DNA (cDNA) in 1:10 dilution using reverse transcriptase MultiScribe (Thermo Fisher Scientific), according to manufacturer protocol.

2.4. Polymerase chain reactions

Polymerase chain reactions (PCR) were performed to amplify the *LWS* opsin gene expressed in the retina of owls, using specific primers designed with Primer 3 (v.0.4.0) (Koressaar & Remm, 2007; Untergrasser et al., 2012), based on the opsin sequences of *Taeniopygia guttata* and *Columba livia* (GeneBank accession numbers: NM_001076702.1 and AH007800.2, respectively). The Table 1 shows the primers used. The parameters used for primer design were: (1) primer size: min 18, opt 20, max 22; (2) primer Tm: min 58, opt 60, max 62; (3) max Tm difference: 2. Other parameters were not altered from Primer3 default sets.

The PCRs were performed with Platinum® Taq DNA Polymerase kit (Life Technologies). The final solution of each reaction was 50 µL, with the reagents $MgCl_2$, with concentration of 50 µM, the primers at 20 µM each and dNTPs − 10 µM each. The enzyme Platinum Taq buffer (1.5 nM), 10x buffer and water were used to complete the reaction

Table 1
Primers designed from *Taenopygia guttata* and *Columba livia LWS* sequences, and their respective annealing temperatures.

Primers	Annealing temperature
LWS_ZFinch164_Fw 5′-AGCGCTGGTTCGTGGTCT-3′	57
LWS_ZFinch164_Rv 5′-GGGAAGAAGCAGCAGGTGAC-3′	
LWS_ZFinch180_Fw 5′-ACATCAAGTTCGACGGGAAA-3′	62
LWS_ZFinch180_Rv 5′-GCTGCCGCTGAACACGTC-3′	
LWS_ZFinch277_Fw 5′-AGCAGAAGGAGTCGGAGTCG-3′	62
LWS_ZFinch277_Rv 5′-TGTAGATGGTGGCGCTCTTG-3′	
LWS_ZFinch308_Fw 5′-TGGTGGTGGTGATGATCTTG-3′	60
LWS_ZFinch308_Rv 5′-GCCGGTTCATGAAGACGTAG-3′	

volume. The following parameters were adopted: initial temperature at 94 °C for 2 min; 37 cycles of (1) 94 °C for 30 s; (2) annealing temperature for 30 s (Table 1) and (3) extension temperature at 72 °C for 30 s; final extension at 72 °C for 7 min. The PCR products were visualized by agarose gel electrophoresis (2%) and purified using *Illustra™ GFX™ PCR DNA and Gel Band purification* kit (GE Healthcare), according to the manufacturer protocol.

The sequencing was performed based on the Sanger methodology (Sanger, Nicklen, & Coulson, 1977), using the Big Dye Terminator v3.1 (Life Technologies) and the 3500xL sequencer (Life Technologies).

2.5. Analysis of genetic data

The obtained *LWS* sequences were aligned using ClustalW algorithm with sequences from other bird species, *Taeniopygia guttata*, *Columba livia* and *Serinus canaria*, with the program BioEdit v7.2.5 (Hall, 1999). The amino acids at spectral tuning sites of the LWS opsin 164, 181, 262, 269, and 292 (following bovine rod numbering – GenBank accession number: NM_001014890.2) were identified. With this result, the LWS opsin spectral absorption peak present in each species was estimated based on literature data.

2.6. Immunohistochemistry

The eyecups of *T. furcata, M. choliba*, *A. cunicularia* and *G. brasilianum* were fixed in paraformaldehyde (PFA) diluted in phosphate buffer saline 0.1 M (PBS), and cryoprotected with 30% sucrose solution diluted in PBS, for 48 h. Radial sections at 12-μm thicknesses were obtained in cryostat (CM1100 Leica, Nussloch, Germany), collected onto gelatinized glass slides and stored at − 20 °C. Retinal sections were incubated overnight with polyclonal antibodies against red/green opsin (Millipore, cat. N. AB5405; 1:200), diluted in 0.1 M PBS with 0.3% Triton X-100. The tissues were incubated in secondary antibody goat anti-rabbit immunoglobulin G (whole molecule; 1:500; Jackson Immunoresearch Laboratories, West Grove, Pa., USA), conjugated with the fluorescent molecule CY3), diluted in 0.1 M PBS with 0.3% Triton. The slides were mounted using Vectashield with 4,6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA), and observed under a fluorescent microscope (LEICA DM5500B). Images were processed using Adobe Photoshop CS3 for adjustment of contrast and brightness.

3. Results

3.1. LWS gene

We amplified and sequenced partial coding sequences of the *LWS* gene expressed in retinas of five owl species: the cathemeral *Athene cunicularia*, the diurnal *Glaucidium brasilianum*, and the nocturnals *Asio clamator, Megascops choliba*, and *Tyto furcata*. Approximately 500 bp were amplified and the λ_{max} of the LWS opsins were estimated based on the amino acid residues in spectral tuning sites (Fig. 1). One specie, *A. cunicularia*, had the amino acid substitution T269A, and an estimated spectral absorption peak at ~537 nm (Fig. 2). For one species, *T. furcata* it was not possible to estimate the λ_{max} of the LWS opsins because we could not amplify the spectral tuning site 261. The other three species had the same amino acids at the spectral tuning sites, A164, H181, Y261, T269, and A292, and estimated absorption peak at 552–554 nm. Table 2 shows the amino acids located at the spectral tuning sites of the LWS photopigment, in each species.

3.2. LWS photoreceptor

Analysis of the retinal sections revealed the presence of L cones in four species of owls, the nocturnal *T. f. tuidara and M. choliba*, the cathemeral *A. cunicularia* and the diurnal *G. brasilianum* (Fig. 3). We observed longer L cone outer segments in the diurnal (Fig. 3A) and cathemeral (Fig. 3B) species, compared to the nocturnal species (Fig. 3C, D).

Comparing qualitatively the amount of labelled cones it was observed that the species from the Strigidae family had a higher density of LWS cones comparing to the specie from the Tytonidae family (*T. furcata*).

4. Discussion

In this study, we aimed to investigate the critical sites for the LWS opsin spectral tuning, 164, 181, 261, 269, and 292 (Yokoyama & Radlwimmer, 1998), in five owl species, *Asio clamator, Glaucidium brasilianum, Megascops choliba, Athene cunicularia*, and *Tyto furcata*, using genetic sequencing, and to identify the photoreceptor population containing the LWS photopigment. Our results showed the same amino acid combination at the spectral tuning sites (A164, H181, Y261, T269, and A292) in three species, *A. clamator, G. brasilianum*, and *M. choliba* with the absorption peak at 555 nm, and the substitution T269A in the cathemeral species *A. cunicularia*, causing an estimated blue-shift of the absorption peak at ~537 nm. We were also able to identify L cones in retinas of four species and we suppose the existence of a difference in the number of LWS cone comparing the retina of different owl's families (Strigidae and Tytonidae).

According to Yokoyama, Blow, and Radlwimmer (2000), the most commonly present amino acid in position 164 in birds is serine, which was found in pigeon (Kawamura, Blow, & Yokoyama, 1999), chicken (Bowmaker & Knowles, 1977) and zebra finch (Yokoyama et al., 2000). However, our results show that, for the five owl species we analyzed, the amino acid alanine, instead of serine, is present at this site. At other sites (181, 261, 269, and 292), the same amino acids described in other birds (HYTA, respectively) were observed, except for *A. cunicularia* that showed a T269A substitution and *T. furcata* that we could not estimate the site 261. In the case of *T. furcata* our experiments not amplified the segment responsible to express the 261. We suppose that the LWS gene has a low expression in the retina or our primers were not specific enough to amplify the cDNA of this specie.

In birds and mammals, the substitution of a serine for an alanine at site 164 is responsible for a spectral shift of approximately 7 nm toward shorter wavelengths (Hart, 2001; Yokoyama & Radlwimmer, 1998). In birds, for the combination of amino acids SHYTA, Yokoyama and Radlwimmer (2001) described a photopigment with a spectral absorption peak between 559 and 561 nm. The substitution of a tyrosine for an alanine at site 269 causes a 15 nm shift toward shorter wavelengths (Yokoyama & Radlwimmer, 1998). This change forms the combination of amino acids AHYAA, with a spectral absorption peak around 537 nm (Yokoyama & Radlwimmer, 2001).

Accordingly, we may infer that the individuals of the specie *A. cunicularia* with T269A substitution should show a spectral absorption peak of the LWS opsin at around 537 nm. Intriguingly, the same change was found in one owl species studied by Wu et al. (2016), the long-ear owl (*Asio otus*) – a mainly nocturnal owl. In the same study, other specie of the same genera *Athene (A. clamator)* did not show this amino acid change. A variation in the 269 site between owl's species can be occurring independently of phylogeny. For the other three species of owls, the spectral absorption peak should be at around 552 and 554 nm. This value is close to that described for the LWS opsin in another owl species, *Strix aluco* (555 ± 3 nm), measured by microspectrophotometry (Bowmaker & Martin, 1978). Studies on the genetics of the LWS photopigment of *S. aluco*, as well as microspectrophotometry studies of the visual photopigments of the five owl species investigated here should be performed to confirm data on genetics and LWS peak absorption. To predict the data about absorption peak using genetic analysis is necessary to have previous studies measuring the absorption peak of opsins. According to Bloch (2016), it could be quite complicated to predict spectral tuning based on amino acids residues. However, differences in the sequence of opsin genes across birds do not always translate into

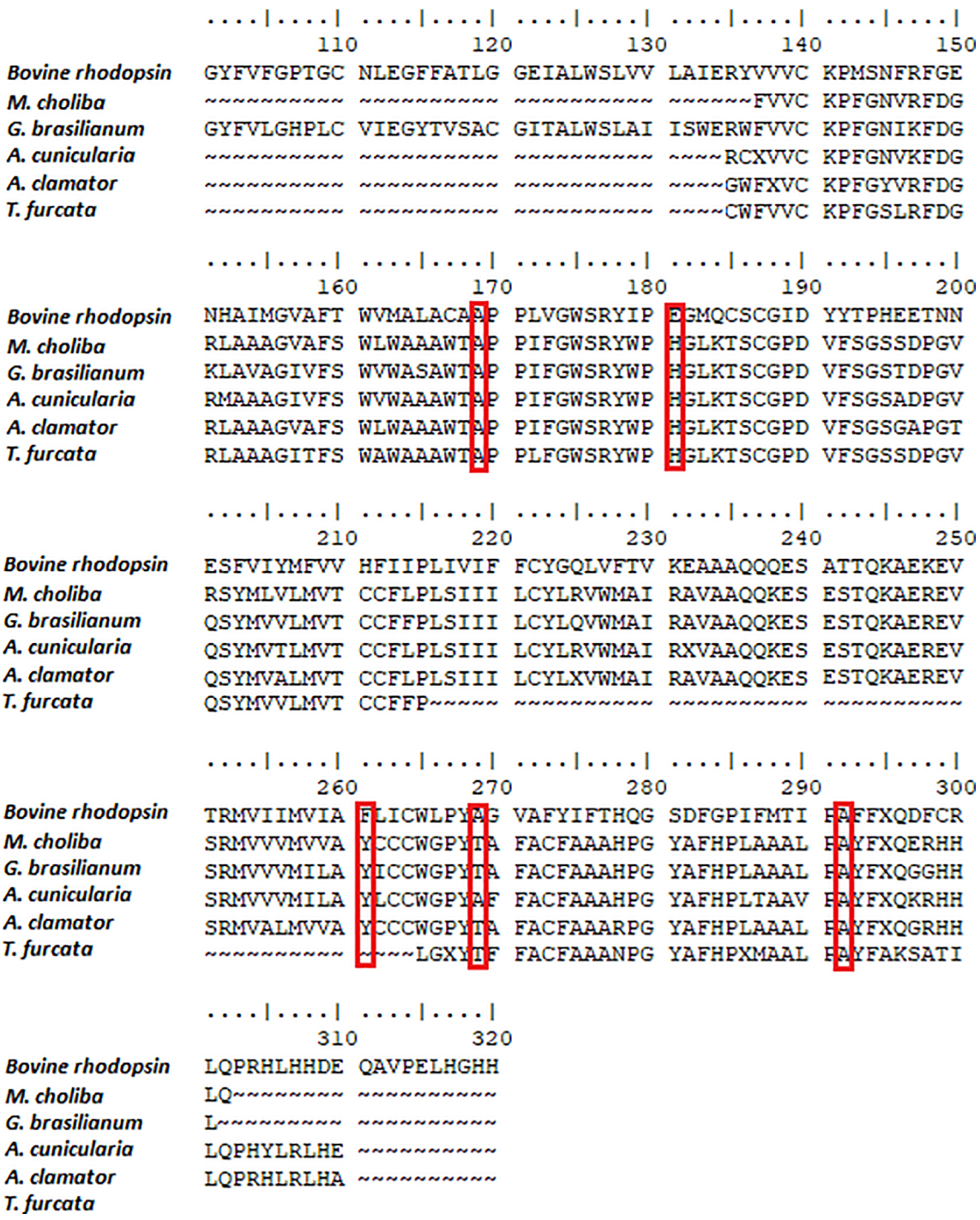

Fig. 1. Sequencing alignment of the amino acids of LWS opsin of *Athene cunicularia*, *Glaucidium brasilianum*, *Asio clamator, Megascops choliba and Tyto furcata*, and the LWS opsin of the birds *Sturnus vulgaris* and *Taenopygia guttata* (GenBank accession numbers: XM014893396, NM001076702), and the bovine rhodopsin (NM_001014890.2).

spectral shifts, as it can be seen in warblers SWS2 opsin – two residues (49 and 269) vary without significant changes in the λ_{max} (Bloch, Morrow, Chang, & Price, 2015). On the other hand, studies with green anole and goldfish *SWS2* (Yokoyama & Tada, 2003), human (Asenjo, Rim, & Oprian, 1994) and bovine (Chan, Lee, & Sakmar, 1992) *LWS* found changes up to 12 nm in the protein absorption peak. Table 3 shows amino acid combination and the respective spectral absorption peak in birds and other organisms. Although the idea of compare opsins from different species in order to predict spectral tuning out of known substitutions has become a common practice, there is a need of cautious when comparing opsins that carry different substitutions at the site of interest or if they differ at many amino acid sites. (Ward et al., 2008; Hofmann et al., 2009).

Among the possible amino acid variations for the spectral tuning of the LWS opsin, it is observed that most birds have a spectral absorption peak above 560 nm, with a mean of 565 nm (Hart & Hunt, 2007). Few species fall below of this value. Among them are the penguin, blackbird and owls. In the penguin retina (*Spheniscus humboldti*), Bowmaker and Martin (1985) identified by microspectrophotometry a type of cone with a peak of spectral absorption at 543 nm, shifted towards wavelengths shorter when compared to other birds. This displacement has been interpreted as due to adaptations for aquatic environment, where

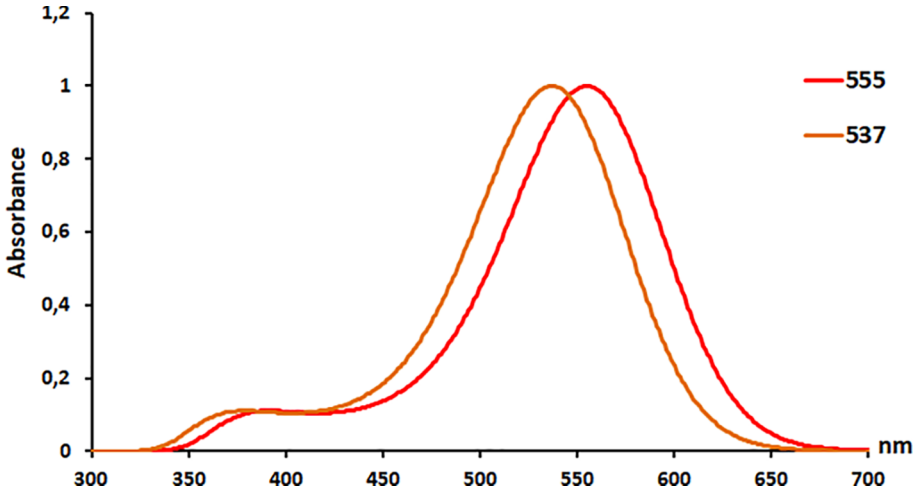

Fig. 2. Spectral absorption curves of the LWS opsins of *Athene cunicularia* (5 3 7) and of *Glaucidium brasilianum*, *Asio clamator* and *Megascops choliba* (5 5 5), predicted based on the amino acids located at spectral tuning sites. Spectral absorption curves were estimated according to Stockman et al. (2000).

Table 2
Amino acids found in the five critical sites for LWS photopigment spectral tuning.

Species/Sites	164	181	261	269	292	λ_{max} (nm)
Asio clamator	A	H	Y	T	A	555
Glaucidium brasilianum	A	H	Y	T	A	555
Megascops choliba	A	H	Y	T	A	555
Athene cunicularia	A	H	Y	A	A	537
Tyto furcata	A	H	*	T	A	?

A, Alanine; H, Histidine; Y, Tyrosine; T, Threonine. * not evaluated.

long wavelengths do not prevail (Stonehouse, 1975). The blackbird (*Turdus merula*), has a visual pigment that absorbs long wavelengths with a spectral absorption peak at 557 nm. Although the wavelength maximally absorbed by its photopigment is shifted towards shorter wavelengths, as in the owls and penguin, the oil droplet located between the inner and outer segments changes the effective spectral sensitivity of the cone to about 600 nm (Hart, Partridge, Cuthill, & Bennett, 2000).

The owl *Strix aluco* is among the species known to date to have the LWS visual pigment tuned towards shorter wavelengths (555 nm) and the Great Horned Owl (*Bubo virginianus*), which had a λmax at 555 nm estimated through electroretinogram (Bowmaker & Martin, 1978; Hart et al., 2000; Jacobs et al., 1987). In the case of owls, this shift towards shorter wavelengths might be attributed to owls having nocturnal ancestors.

From molecular and phylogenetic studies, it is known that the ancestor of birds had a serine at site 164 (S164). The common ancestor of owls, in turn, had an alanine at this site (S164A), along with twilight preferential activity (Heesy & Hall, 2010; Wu et al., 2016). Many living owls currently, such as those studied by Wu et al. (2016), are nocturnal. However, some species are adapted to daylight – this is the case of *Athene cunicularia* and of *Glaucidium brasilianum*. Despite the differences observed among owls, three species studied have maintained the *LWS* gene with the same amino acid sequence (AHYTA, 164, 181, 261, 269, and 292 respectively), a fact that differs from that described in snakes. In snakes, whose ancestors are nocturnal, it is observed that, among different species, the amino acids present at the five sites important for the spectral tuning of opsin differ between nocturnal and diurnal animals, presenting the AHYAA (537 nm, nocturnal), AHFTA (543 nm, diurnal), AHYTA (553 nm, diurnal and nocturnal) and SHYTA (560 nm, diurnal) (Hauzman, Bonci, Suárez-Villota, Neitz, & Ventura, 2017). It is possible that in owls there was not sufficient evolutionary time to promote a selective pressure on opsin sequence with the change in the habits. It is also possible that other aspects of avian vision constrain the evolution of LWS and/or the other opsins, limiting its divergence within the clade.

The absence of difference among the LWS opsin of these owl species in the five critical sites studied is not related with their habit differences. Although not critical, changes in other amino acids can affect the protein structure and functions. In addition, since the circadian pattern is influenced by clock genes (Chakir et al., 2014), comparative studies based on transcripts and protein levels might contribute to reveal specific expression variations in diurnal and nocturnal animals. Genetic analysis of other opsins is one step to investigate impact of circadian activity on vision. However, complex organism changes along time are influenced by numerous different factors, which is why one cannot elect just one of those factors in order to justify a behavior characteristic. It is also possible that hormonal differences among species could interfere with the habits of these animals (Cassone, Paulose, Harpole, Li, & Whitfield-Rucker, 2015). Additionally, electrophysiological studies that would evaluate neuronal activity and bring information to investigate molecularly and cellularly the processes that govern these signals (Carter & Shieh, 2015), as well as the impact of the illumination on the circadian rhythm (Brown, 2016), are also important in the investigation of the connection between circadian activity and vision.

This is the first study to show the L cones labeled in retinas of owls. We could identify the LWS pigment in a subpopulation of cones in the retina of four species of owls, three from the Strigidae family and one from the Tytonidae family. We observed a reduction in the number of L cones in the Tytonidae species, which should be confirmed by measurement of density and distribution of this cone type. Difference in cell density among different owl's families was reported by Lisney et al. (2012) that investigated the density of retinal ganglion cells. The authors showed a smaller density in the ganglion cell in the Tytonidae species studied compared to other owls. Since nocturnal and diurnal Strigidae seem to have similar cell densities, the difference found for the American barn owl might be related to the phylogenetic distance from Strigidae owls, although further investigation is needed to confirm this hypothesis.

5. Conclusions

The opsin LWS gene was identified in five species of owls (*Athene cunicularia*, *Asio clamator*, *Megascops choliba*, *Glaucidium brasilianum* and *Tyto furcata*), and the respective λmax was estimated in 555 nm for *M. choliba*, *G. brasilianum* and *A. clamator,* and in 537 nm for *Athene cunicularia*. The successful identification of the L cone by immunohistochemistry showed that there is a difference in the L cone

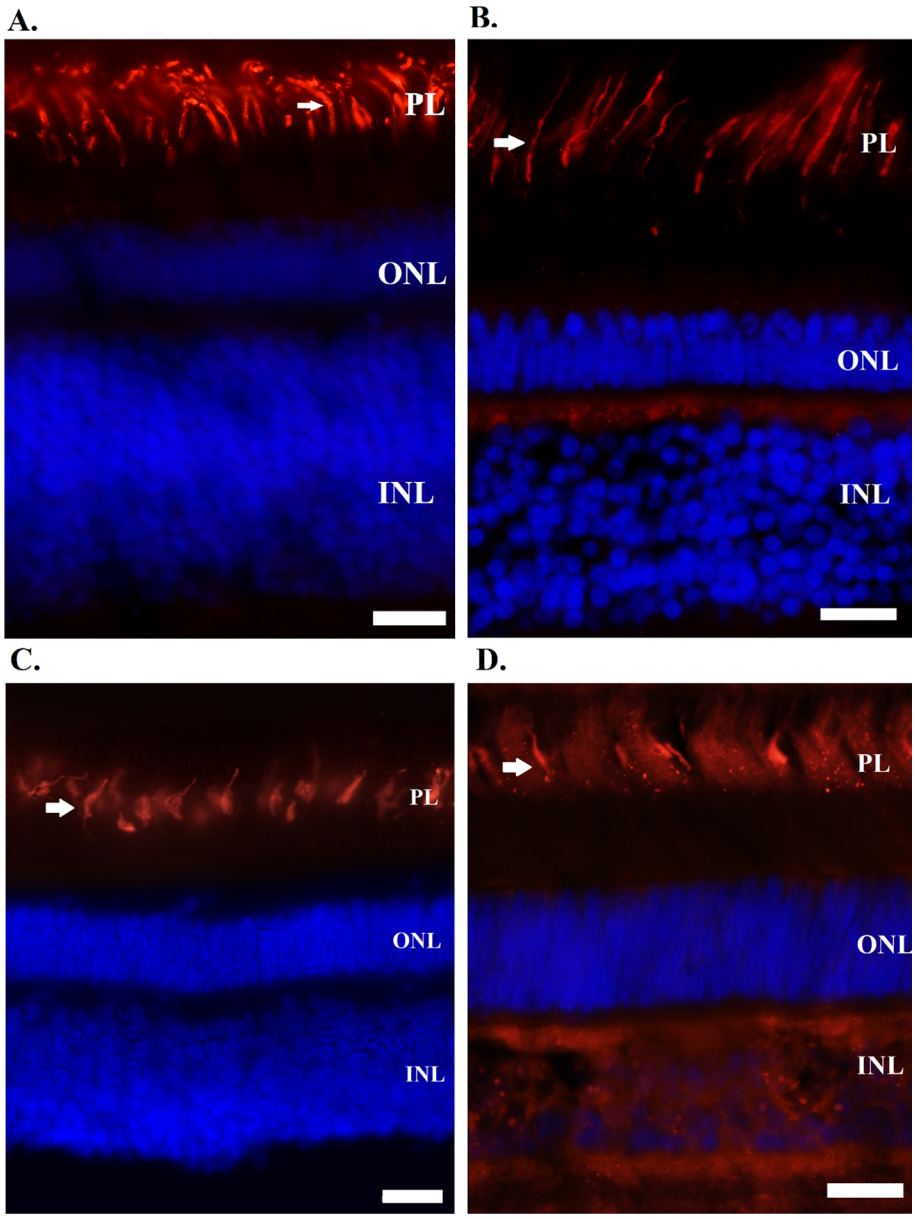

Fig. 3. Retinal radial sections of the diurnal *G. brasilianum* (A), the cathemeral *A. cunicularia* (B) and the nocturnal *M. choliba* (C) and *T. furcata* (D), labeled with antibody against L opsin (red). The arrows point to the outer segments of L cones revealed in red. Cells nuclei labeled by DAPI (blue). PL, photoreceptor layer; ONL, outer nuclear layer; INL, inner nuclear layer. Scale bars = 20 μm.

Table 3
Amino acid combination and the respective spectral absorption peak in birds and other organisms.

Species	Animal	Absorption peak	Aa combination	References
Turdus merula	Blackbird	557 nm (msp)	–	Hart, 2001
Anas platyrhynchos	Duck	567 nm (msp)	–	Jane & Bowmaker, 1988
Gallus gallus domesticus	Chicken	570 nm (msp)	SHYTA	Bowmaker et al., 1997; Yokoyama and Radlwimmer, 2001
Erythrolamprus poecilogyrus	Aesculapian Coral Snake	553 nm (i.b.s)	AHYTA	Hauzman et al., 2017
Homo sapiens	Human	558 nm (msp)	SHYTA	Dartnall, Bowmaker, & Mollon, 1983; Yokoyama and Radlwimmer, 2001
Spheniscus humboldti	Humboldt penguin	543 nm (msp)	–	Bowmaker & Martin, 1985
Bubo virginianus	Great Horned Owl	555 nm (erg)	–	Jacobs et al., 1987
Strix aluco	Tawny owl	555 nm (msp)	–	Bowmaker & Martin, 1978
Megascops choliba	Tropical screech owl	555 nm (i.b.s)	AHYTA	This study
Asio clamator	Sriped owl	555 nm (i.b.s)	AHYTA	This study
Glaucidium brasilianum	Ferruginous pygmy owl	555 nm (i.b.s)	AHYTA	This study
Tyto furcata	American barn owl	555 nm (i.b.s)	AHYTA	This study
Athene cunicularia	Burrowing owl	537 nm (i.b.s)	AHYAA	This study

erg – electroretinogram; msp – microspectrophotometry; i.b.s – inferred by sequencing

density related to the owĺs phylogeny. Examination of the retinal topography of these species will therefore be relevant to further investigate the phylogenetic differences and the relationship between habits and visual adaptations.

6. Contributors

FTGRV and MJVN had the same contribution to the present paper, sharing the first author position. FTGRV, DMOB, MJVN, and EH contributed to the study design, data analysis, data interpretation, literature search and writing the manuscript. JB contributed to the study design, ethics committee approval, technical support and data interpretation. DV contributed to the study design and data interpretation. All the authors approved the manuscript.

7. Ethics approval

Comissão de Ética no uso de animais (UFMG – 39/2011).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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