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Photoreceptors morphology and genetics of the visual pigments of *Bothrops jararaca* and *Crotalus durissus terrificus* (Serpentes, Viperidae)

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ABSTRACT

Snakes inhabit a great variety of habitats, whose spectral quality of light may vary a lot and influence specific adaptations of their visual system. In this study, we investigated the genetics of the visual opsins and the morphology of retinal photoreceptors, of two nocturnal snakes from the Viperidae family, *Bothrops jararaca* and *Crotalus durissus terrificus*, which inhabit preferentially the Atlantic Rain Forest and the Brazilian Savannah, respectively. Total RNA was extracted from homogenized retinas and converted to cDNA. The opsin genes expressed in snake retinas, *LWS*, *RH1*, and *SWS1*, were amplified by polymerase chain reactions (PCRs) and sequenced. The absorption peak (λ_{max}) of the opsins were estimated based on amino acids located at specific spectral tuning sites. Photoreceptor cell populations were analyzed using immunohistochemistry with anti-opsin antibodies. Results showed the same morphological cell populations and same opsins absorption peaks, in both viperid species: double and single cones with LWS photopigment and λ_{max} at ~555 nm; single cones with SWS1 photopigment and λ_{max} at ~360 nm; and rods with the rhodopsin RH1 photopigment and λ_{max} at ~500 nm. The results indicate adaptations to nocturnal habit in both species despite the differences in habitat, and the possibility of a dichromatic color vision at photopic conditions.

1. Introduction

In most vertebrates vision is a sensory modality that prevails over others and provides a major way to access environmental information for biological functions and behaviors, such as finding a food source, mating partners, and avoidance of predators. A number of adaptive specializations can be observed in the visual structures, associated with specific ecological niches of the species. The proportion of rod and cone photoreceptors in the retina, for instance, can be related to nocturnal or diurnal habits, as shown by the lack of cones in night-dwelling geckos, and the absence of rods in strictly diurnal chameleons and snakes (Bowmaker, Loew, & Ott, 2005; Hauzman, Bonci, Suárez-Villota, Neitz, & Ventura, 2017; Walls, 1942). The wavelength absorption peak (λ_{max}) of the opsins is also variable among species. Many studies described adjustments of the spectral tuning to optimize photon capture in the environment inhabited by the species (Bowmaker & Hunt, 2006; Lythgoe, 1979).

In snakes, a number of morphological singularities of the photoreceptors are observed, such as the emergence of a unique type of double cone, the absence of cone-like photoreceptors in strictly nocturnal colubrids, and the loss of a typical rod-like photoreceptor in diurnal species (Bhattacharyya, Darren, Schott, Tropepe, & Chang, 2017; Hart, Coimbra, Collin, & Westhoff, 2012; Hauzman et al., 2014; 2017; Sillman, Govardowskii, Röhlich, Southard, & Lowe, 1997; Simões et al., 2016; Schott et al., 2016; Underwood, 1967; Walls, 1942; Wong, 1989). In most snake species, three opsin genes are expressed in the retinas, the long wavelength sensitive cone opsin gene *LWS*, the short wavelength sensitive cone opsin gene *SWS1*, and the rhodopsin gene *RH1* (Davies et al., 2009; Hauzman et al., 2017; Simões et al., 2016). Despite the absence of typical rod-like photoreceptor, diurnal colubrids express the rhodopsin gene *RH1* in a cone-like photoreceptor (Bhattacharyya et al., 2017; Hauzman et al., 2017; Schott et al., 2016) on the other hand, some nocturnal colubrids with all-rod retinas have cone visual pigments (Simões et al., 2016).

In the Viperidae family, a typical duplex retina composed by two classes of cones amongst a majority of rods was described for a few species (Walls, 1942), maintaining the same retinal structure observed in species from the Henophidia ("basal") group, such as *Python regius*

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(Sillman, Carver, & Loew, 1999) and *Boa constrictor imperator* (Sillman, Johnson, & Loew, 2001). This pattern is also found in most placental mammals, which also have rod-dominated retinas, lack oil droplets within their photoreceptors, and have lost the *RH2* and *SWS2* opsin genes. Those features may suggest evolutionary adaptations to nocturnal/mesopic conditions (Davies, Collin, & Hunt, 2012).

Among viperids, different species inhabit distinct environments, such as open fields or dense forests, and thus are exposed to different photic conditions. The dense foliage has an effect on the spectral quality of the scene, where light with longer wavelengths prevails (Veilleux & Cummings, 2012). The comparison of the visual system of viperid species that belong to the same subfamily but inhabit different habitats may offer a convenient opportunity to assess the adaptive influence of the environment in the structures of the visual system.

In this study, we compared two aspects of the visual system of two Viperidae species, from the Crotalinae subfamily, *Bothrops jararaca*, and *Crotalus durissus terrificus*, which inhabit preferentially the Brazilian Atlantic Rain Forest and the Brazilian Savannah, respectively. We investigated the nucleotide sequences of the opsin genes expressed in the retinas and estimated the spectral absorbance peak (λ_{max}) of their visual pigments based on amino acid composition at spectral tuning sites. We also performed a morphologic analysis of the photoreceptors in which those pigments are expressed. The results suggest adaptation to nocturnal habit in both species and the similarities of the data indicate that the phylogenetic proximity of both species may prevail over specific environmental adaptations.

2. Materials and methods

2.1. Animals

The retinas of three individuals of each species, *Bothrops jararaca*, and *Crotalus durissus terrificus*, provided by the Butantan Institute, São Paulo, SP, Brazil were used. Euthanasia was performed with the injection of a lethal dose of Thiopental (100 mg/kg). Animal procedures were in accordance with the Ethical Principles of Animal Experimentation, established by the Brazilian Animal Experimentation College (COBEA). This project was approved by the Animal Research Ethics Committee of the Psychology Institute, University of São Paulo (1805090417).

2.2. Opsin genetics

2.2.1. Total RNA isolation and complementary DNA synthesis

Eyes of one subject of each species were enucleated and stored in RNAlater® (Life Technologies, Carlsbad, CA, USA), at 4 °C. Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA, USA), according to manufacturer's protocols. A 1:10 RNA dilution was prepared with 500 ng oligo-dT (12-mer) primer and converted to complementary DNA (cDNA) using the reverse transcriptase enzyme MultiScribe™ (Applied Biosystems, Foster City, California, USA), according to the manufacturer's protocol.

2.2.2. cDNA amplification with polymerase chain reaction (PCR) and sequencing

PCRs were performed to amplify partial sequences of the three opsin genes expressed in snake retinas. Specific primer pairs were obtained from previous studies (Hauzman et al., 2017), or designed using Primer 3 (v.0.4.0) (Untergrasser et al., 2012) (Supplementary Table S1). PCRs were carried out using High Fidelity Platinum Taq Polymerase, 10x High Fidelity Buffer and $MgCl_2$, 10 mM GeneAmp dNTPs (Life Technologies, Carlsbad, California, USA) and 20 μM primers in 50 μL reactions. The PCR conditions were: 1) denaturation at 94 °C for 1 min; 2) 37 cycles at 94 °C for 15 s, varied annealing temperature for 30 s (Supplementary Table S1), extension temperature at 72 °C for 30 s; and 3) final extension temperature at 72 °C for 10 min.

PCR products were visualized by electrophoresis in agarose gel at 1.0% and kept in −20 °C. Purification was performed with *Illustra GFX™ PCR DNA and Gel Band Purification Kit* (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Sequencing was performed with Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), in a 3500 Applied Biosystems Sequencer. Sequences were analyzed with BioEdit v7.2.5 (Hall, 1999).

2.2.3. Spectral tuning estimation of the opsins

To estimate the opsins spectral absorption peaks (λ_{max}), we identified the amino acids located at spectral tuning sites previously described for other vertebrates. The amino acids of the three opsin genes were numbered based on the bovine rhodopsin sequence (GenBank accession number: NM001014890). To estimate the λ_{max} of the rhodopsin RH1 photopigment, we analyzed the amino acids located at residues 83, 122, 207, 265, 269, 285, and 292 (Hunt, Dulai, Partridge, Cottrill, & Bowmaker, 2001; Yokoyama, 2000; Yokoyama, Tada, Zhang, & Britt, 2008). The spectral peak of the LWS photopigment was estimated based on the amino acids located at five residues: 164, 181, 261, 269, and 292 (Yokoyama & Radlwimmer, 1999). The presence of the amino acids Serine, Histidine, Tyrosine, Threonine, and Alanine in those sites, is known to generate an LWS with λ_{max} at ~560 nm in different vertebrate lineages, and the substitutions S164A, H181Y, Y261F, T269A, and A292S are generally responsible for downward shifts of 7, 28, 8, 15 and 27 nm, respectively (Yokoyama & Radlwimmer, 1999). The *SWS1* gene expresses photopigments sensitive to short wavelengths from ultraviolet to the violet band. The presence of the amino acid Phenylalanine at residue 86 generates a λ_{max} at the UV band, around 360 nm (Cowing et al., 2002; Fasick, Applebury, & Oprian, 2002; Hunt et al., 2007) in non-avian and non-Primates SWS1 photopigments (Carvalho, Davies, Robinson, & Hunt, 2012; Hunt et al., 2007). We thus, analyzed the amino acid located at residue 86 to estimate the sensitivity at the UV or violet band in the viperid snakes. Spectral sensitivity curves were generated using a standard A1-based rhodopsin template (Govardovskii, Fyhrquist, Reuter, Kuzmin, & Donner, 2000).

2.3. Morphologic analysis of photoreceptors in retinal radial sections.

For morphological analysis, the eyes of two specimens of each species were collected, the corneas and lenses were removed, and eyecups were fixed in paraformaldehyde (PFA) 4%, diluted in phosphate buffer (PB) 0.1 M, pH 7.4, for 3 h. Eyecups were cryoprotected in 30% sucrose solution, diluted PB 0.1 M, for 24 h, embedded in Tissue-Tek OCT (Sakura Finetechnical Co. Tokyo, Japan), and sectioned at 12 μm thickness. Sections were obtained at −25 °C in cryostat Leica CM1100 (Nussloch, Germany), collected into gelatinized slides, and kept at −20 °C until use.

2.3.1. Immunohistochemistry procedures

Radial sections were pre-incubated for 1 h in 10% normal goat serum (Sigma-Aldrich, St. Louis, MO, USA), diluted in PB 0.1 M with Triton X-100 at 0.3%. Tissues were incubated overnight with polyclonal antibodies against blue opsin (Chemicon International, Hofheim, Germany, cat. n. AB5407; 1:300) and against red/green opsin (Chemicon International, cat. N. AB5405; 1:500), diluted in 0.1 M PB with 0.3% Triton X-100. Previously, the same antibodies had been successfully used to label S and L/M cones in snake retinas (2017; Hauzman et al., 2014), and the specificity of both antibodies for snake retinas had been described (Hauzman et al., 2014). Sections were washed in PB 0.1 M with 0.3% Triton X-100, and incubated with secondary antibody goat anti-rabbit immunoglobulin G (whole molecule; Jackson Immunoresearch Laboratories, West Grove, Pa., USA), conjugated to fluorescein isothiocyanate (FITC), diluted in PB 0.1 M with Triton 0.3%, in a 1:200 dilution, for 2 h. Slides were washed in PB 0.1 M and mounted with Vectashield with 4,6-diamidino-2-phenylindole

(DAPI; Vector, Burlingame, CA). Secondary antibody specificity was assessed by tissue incubation with a buffer solution without primary antibodies. For labeling rods, retinas were incubated with FITC-conjugated wheat germ agglutinin (WGA, Vector Labs, Burlingame, CA) in a 1:6000 dilution.

Slides were observed in a fluorescence microscope (DRMXE, Leica Microsystems GmBH, Germany), with a 40x objective lens, equipped with a digital camera (DSR1, NIKON, Japan), connected to a computer. Images acquisition were made with the program NIS Elements AR (NIKON, Japan). For each photographed field, emission channels were captured separately and then merged into one single image with the program Photoshop CS6 (Adobe Systems).

3. Results

3.1. Opsin genetics

We amplified and sequenced partial coding sequences (~800 bp) of the three opsin genes, *LWS*, *RH1*, and *SWS1*, expressed in retinas of the viperid snakes *B. jararaca* and *C. d. terrificus* (GenBank accession numbers: MK582577 - MK582582).

The λ_{max} of the LWS, RH1, and SWS1 opsins were estimated based on the amino acid residues at spectral tuning sites (Fig. 1), and no differences in those amino acid compositions were observed between both species. Based on the presence of the amino acids D83, E122, M207, W211, H265, A292, and A295 in the rhodopsin photopigments, we predicted a λ_{max} at ~500 nm (Fig. 2). The presence of the amino acids A164, H181, Y261, T269, and A292, in the spectral tuning sites of the LWS photopigment of both species, may lead to a λ_{max} at ~553 nm (Fig. 2). Based on the presence of the residue F86 in the SWS1 photopigment, we estimated a peak absorbance in the ultraviolet spectrum, at ~360 nm (Fig. 2), in both species.

3.2. Morphological analyses

Analyses of the retinal sections showed a population of rods stained by WGA (Fig. 3E, F). Single and double cones sensitive to medium/long wavelengths were identified with the antibody against L/M opsins (Fig. 3A, B). Small single cones sensitive to short wavelengths were identified with the antibody against S opsin (Fig. 3C, D).

4. Discussion

The poorly explored diversity and adaptations of the visual system of snakes provides an opportunity to complement visual ecology studies. As observed in other snakes from the Henophidia and Caenophidia groups, the Viperidae species approached in this study express three visual pigment genes, *LWS*, *RH1*, and *SWS1*. The loss of the two other opsin genes, *RH2* and *SWS2* in snakes, is believed to be associated with a nocturnal/crepuscular and fossorial phase of ancestral lineages (Davies et al., 2009), as it was suggested for mammals, which passed through a nocturnal/mesopic “bottleneck” (Walls, 1942).

Results from our morphological analysis agree with previous descriptions of retinas from the Viperidae snakes *Causus rhombeatus* and *Bitis arietans*, with a population of rods and three distinct types of cones: double cones, large single cones, and small singles cones (Walls, 1942). Here, we identify small cones as sensitive to short wavelengths, containing the SWS1 opsin, and both double cones and large single cones, containing the LWS opsin, sensitive to medium/long wavelengths.

The retina of viperid snakes differs from that of henophidian (i.e. pythons and boas) (Sillman et al., 1999, 2001) by the presence of double cones, with a large principal member and a small accessory member. However, similarities are observed between these two groups, with adaptation to nocturnal habits, such as the predominance of rods and the presence of UV sensitive cones (Davies et al., 2009; Sillman et al., 1999, 2001; Simões et al., 2016). These retinal features also observed in nocturnal mammals (Joesch & Meister, 2016; Veilleux & Cummings, 2012) is a robust and versatile example of adaptive convergence.

The lack of behavioral or electrophysiological studies maintains unanswered the question concerning the possibility of a dichromatic color vision in snakes, which may be enabled by the presence of two cone populations. It would also be interesting to speculate about the existence of a post-receptoral rod-cone opponency at mesopic light levels, as already reported for rodents (Joesch & Meister, 2016). This would further extend the already known versatility of the duplex retina that allows vision in different light levels (Boynton, 1979). It is also possible that the rod system with λ_{max} at ~500 nm could fill a cone inactivity gap, irrespective of color discrimination. Thus, there is a particular interest in evaluating if there is any transmuted nature in those rods, which could play the role of a cone in mesopic light levels. Based on our morphologic and spectral absorbance data, it is not possible to point to traces of transmutation, as it had been observed in colubrid snakes (Walls, 1942) so that the nocturnal/crepuscular habit seems to be the ancestral state of viperids. Electron microscopy would offer more clues concerning the morphology of their rods and add information about their functional potential.

The predicted spectral absorption peak of the rhodopsin of the two viperid species at ~500 nm, agrees with direct measurement through mass spectrophotometry of the closely related *Crotalus viridis helieri* (Crescitelli, 1956) and with the estimates based on the genetic sequencing of *Bothrops atrox* (Katti, Stacey-Solis, Coronel-Rojas, & Davies, 2018). Simões et al. (2016) estimated the opsins absorption peak of three Viperid species from the Viperinae subfamily, *Bitis nasicornis*, *Echis ocellatus, and Causus rhombeatus*, and estimated the rhodopsin λ_{max} between 491 and 496 nm. This blue shift was attributed to the substitution D83N, which is known to cause a ~6 nm shift of the RH1 photopigment (Yokoyama, 2000). MSP recordings of henophidian snakes (Sillman et al., 1999, 2001) showed a blue shift of the rhodopsin with λ_{max} between 493 and 497 nm, also with the D83N substitution (Davies et al., 2009).

The predicted λ_{max} of the LWS opsin of *B. jararaca* and *C. durissus*, at ~553 nm, agrees with that estimated for the viperid *B. atrox* (Katti et al., 2018) and was similar to that predicted for the Viperinae species *B. nasicornis*, *E. ocellatus* and *C. rhombeattus*, at ~555 nm (Simões et al., 2016). This downward shift to medium wavelengths is attributed to the substitution S164A in the viperids analyzed. However, comparative studies based on genetic analysis and MSP data of the LWS of henophidian snakes, revealed an unexpected blue shift of photopigments with the SHYTA profile in the LWS spectral tuning sites, which is expected to generate a 560 nm absorption peak (Davies et al., 2009). These data indicate that other residues may influence the λ_{max} of the LWS in snakes.

Based on the presence of the amino acid phenylalanine at residue 86, we predicted the λ_{max} of the SWS1 opsin in the UV band, around 360 nm (Cowing et al., 2002; Fasick et al., 2002; Hunt et al., 2007). Other sites may have minor effects on the spectral shift of this visual pigment (Cowing et al., 2002; Yokoyama et al., 2008). Other Viperidae and henophidian snakes also have F86, and UV sensitivity was verified by MSP measurements for diurnal and nocturnal snakes from different families (Sillman et al., 2001, 1997, 1999). As UV light causes damage to the retinal tissue, it is expected that the conferred sensitivity at this spectral region displays a relevant role in the visual system (Sillman et al., 2001). It was suggested that UV sensitivity might be useful to detect the reflectance of rodent urine traces (Desjardins, Maruniak, & Bronson, 1973), or might be used to detect pheromones trails (Ford & Low, 1984), complementing chemoreception with vision, which provides more precise spatial information. Despite the fact that these snakes exhibit nocturnal predatory habits, under daylight conditions it is possible that UV discrimination might be used to locate prey traces and thus improve hunting efficiency (Sillman et al., 2001).

Sensitivity to infrared wavelengths is another sensory modality of

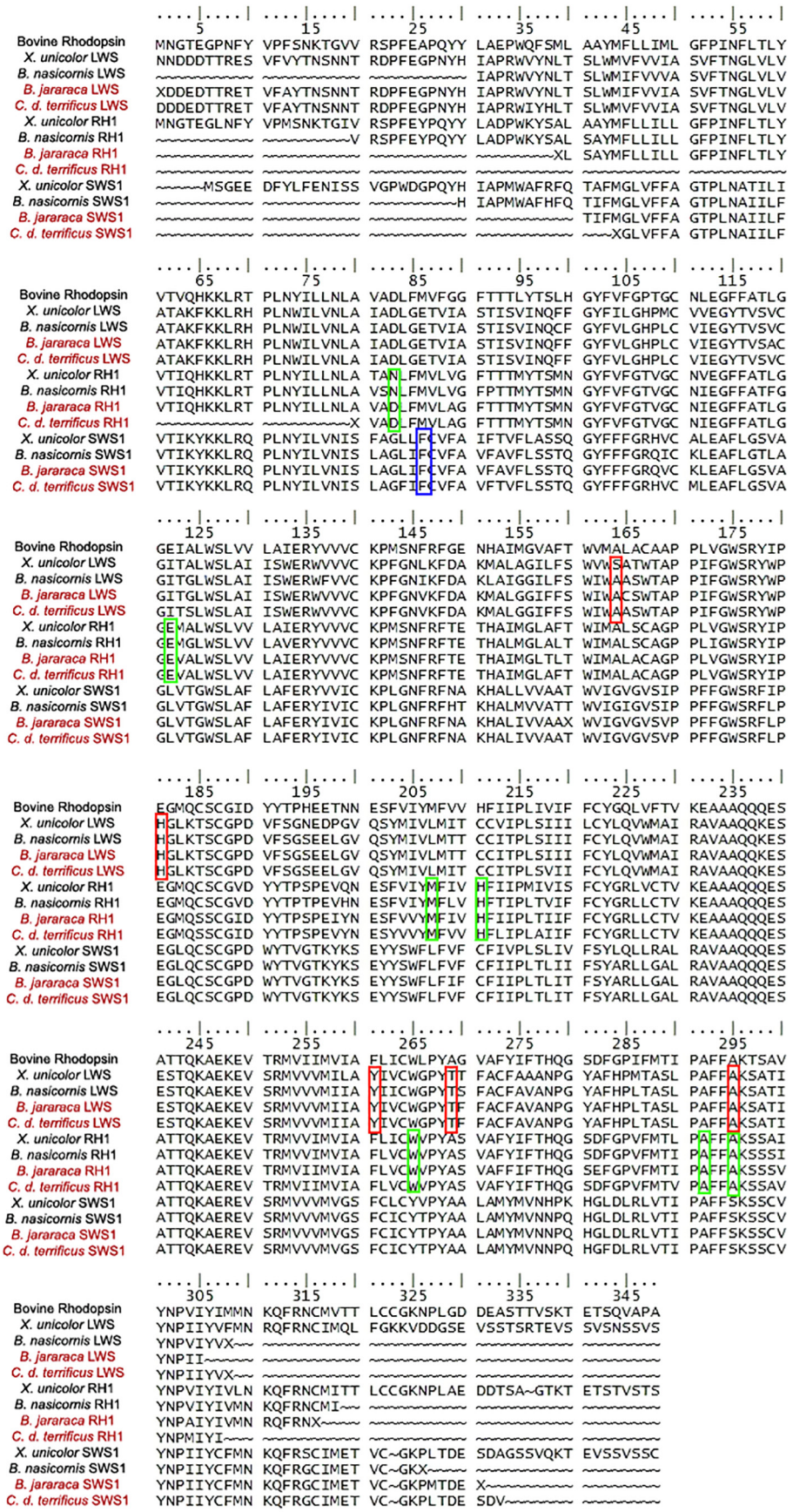

Fig. 1. Sequencing alignment of the amino acids of LWS, RH1, and SWS1 opsins of *B. jararaca* and *C. d. terrificus*, and of the snakes *Bitis nasicornis* (GenBank accession numbers: KX237785.1, KX237873.1, and KX237880.1) and *Xenopeltis unicolor* (FJ497235.1, FJ497233.1, and FJ497234.1), and the bovine rhodopsin (NM_001014890.2). The boxes indicate spectral tuning sites of the RH1 photopigment (green), of the LWS photopigment (red), and of the SWS1 photopigment (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

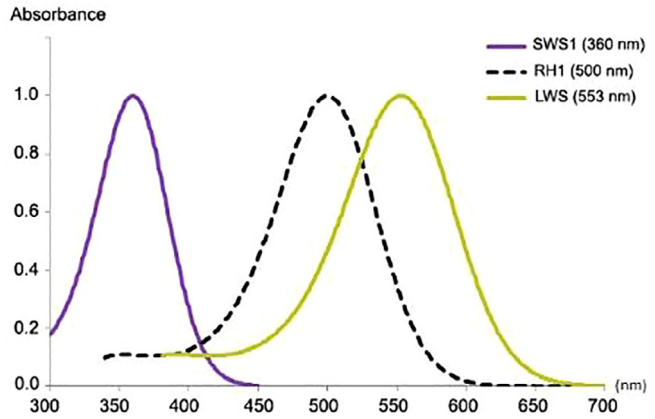

Fig. 2. Spectral absorption curves of the opsins LWS, RH1, and SWS1, of *B. jararaca* and *C. d. terrificus*, predicted based on the amino acids located at spectral tuning sites of each opsin.

Crotalinae snakes, with projections to the visual areas of the brain, creating an overlap with their visual perception (Newman & Hartline, 1982). The ability shared with henophidian snakes suggests that evolutionary pressure related to their ecological niche must be similar. Both species exhibit nocturnal predatory habit and share similar retinal morphology features and opsins spectral tuning peaks. Among the representatives of henophidian and Crotalinae snakes, the presence of double cones containing the LWS visual pigment was the only exclusive feature observed in the retina of viperids (Sillman et al., 1999; Walls, 1942). Double cones function has been proposed to be related to the ability to detect polarized light, but their contribution to behavior is still poorly understood (Cameron & Pugh, 1991).

The comparison between the two species studied showed no difference in the photoreceptor morphology and in the inferred λ_{max} of the opsins, despite the differences in the habitat occupied by the species. However, the direct measurement of the opsins λ_{max} with microspectrophotometry remains to confirm the predicted values and if the habitat does influence the absorption spectra of the opsins of these two species. Furthermore, topographic analyses of cones can show how the distribution of opsins in the retina can be influenced by the different environments as seen in other snake species (Hauzman et al., 2014).

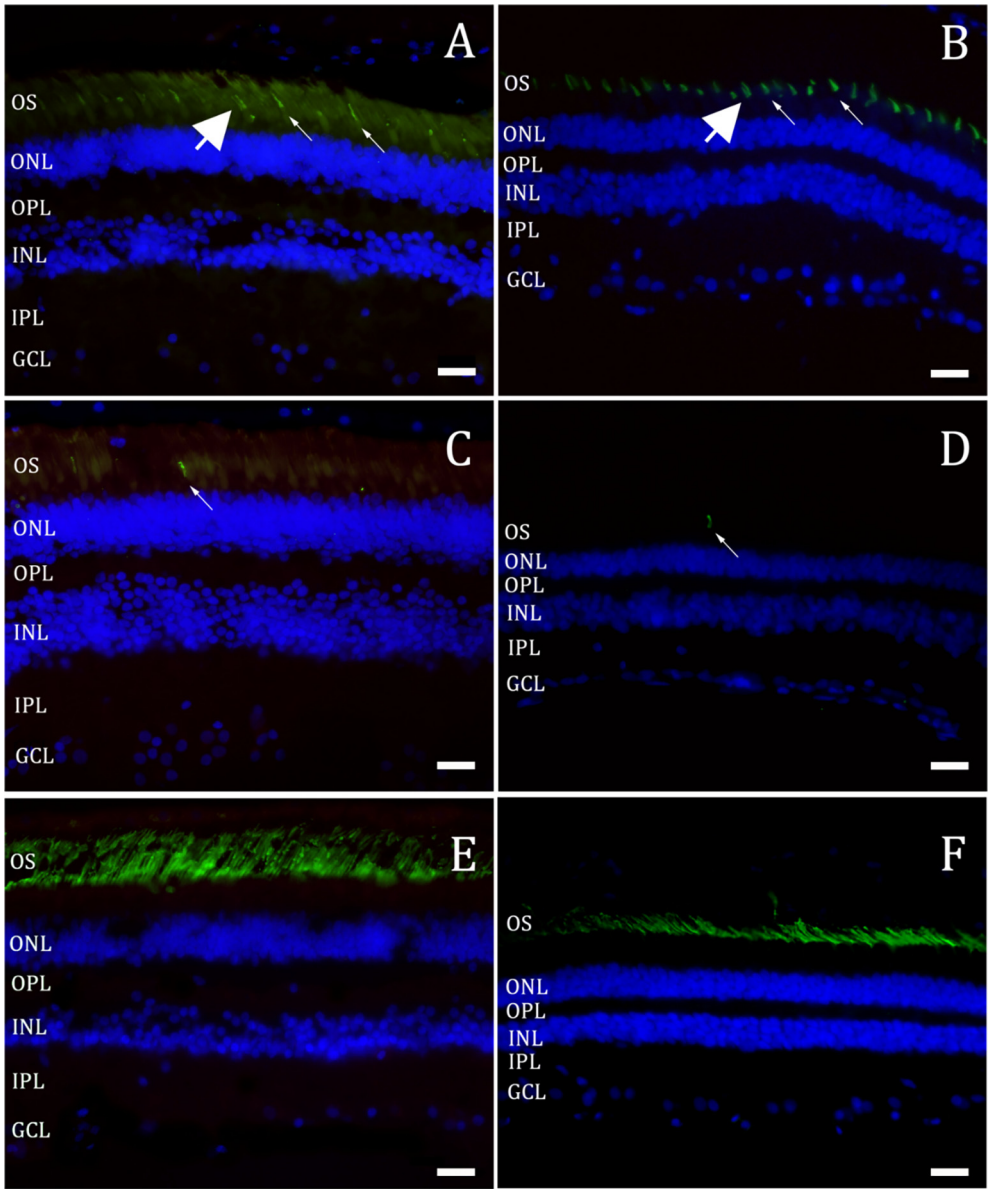

Fig. 3. Radial sections of the retinas of *B. jararaca* (A, C, E) and *C. d. durissus* (B, D, F), labeled with antibodies against L/M opsins (A, B), against S opsins (C, D) and with WGA (E, F). A, B: cone population labeled by the antibody against L/M cones, revealed in green (small arrows: single cones; large arrows: double cones). C, D: cone population labeled by the antibody against S cones revealed in green (small arrows); E, F: rod population labeled by WGA revealed in green. Cell nuclei stained by DAPI in blue. OS, photoreceptor outer segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Declarations of interest

None.

Author's contribution

Bittencourt, G. B. Data collection, data analysis, manuscript drafting.

Hauzman, E. Experiment conception, data collection, data analysis, manuscript revision.

Bonci, D.M.O. Data analysis, manuscript revision.

Ventura, D.F. Data analysis, manuscript revision.

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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.2019.02.006.

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