Chapter 5

Retinal Topographic Maps: A Glimpse into the Animals' Visual World

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Additional information is available at the end of the chapter

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Abstract

The vertebrates' retina has a highly conserved laminar organization of 10 alternating nuclear and plexiform layers. Species differences in the retinal specializations, i.e., areas of higher cell density, among the species, represent specific regions of the visual field of higher importance for a better spatial resolution and indicate distinct evolutionary pressures on the structures of the visual system, which can be related to many aspects of the species evolutionary history. In this chapter, we analyzed the density and distribution of cells of the retinal ganglion cell layer (GCL) and estimated the upper limits of the spatial resolving power of 12 species of snakes from the Colubridae family, 6 diurnal and 6 nocturnal, which inhabit different habitats. Our results revealed lower visual acuity in nocturnal species, compared to diurnal, and we observed different types of retinal specialization, horizontal streak, *area centralis*, or scattered distribution, with higher cell density in different retinal regions, depending on the species. These variations may be related to ecological and behavioral features, such as daily activity pattern, habitat, and substrate preferentially occupied, hunting strategies and diet. This comparative study indicates the complexity of the adaptive strategies of the snakes' visual system.

Keywords: retina, visual ecology, visual acuity, ganglion cells, snakes

1. Introduction

1.1. The visual system

The sensory systems allow the animals to interact properly with their environment and with other organisms. The perception of the surrounding environment is essential for the animals'

survival, and in most vertebrates, the visual system plays a crucial role in basic activities such as foraging behavior, sheltering, flight from predators, and breeding. Functional and anatomical differences of the visual structures often reflect distinct selective pressures implied by the ecological niches.

In all vertebrates, three layers of tissue concentrically arranged form the eyes. The sclera is the outermost layer composed by highly interconnected collagen fibers that support the eye. In the anterior part of the eye, the fibers of the sclera assume an orderly conformation that confers transparency to the sclera, forming the cornea, a lens through which the light can pass. The cornea, together with the crystalline lens, located between the anterior chamber and the vitreous humor, a gelatinous substance that fills the eyeball, enable the focusing of the image in the retina. The second layer is the uvea, formed by the iris, ciliary body, and choroid, and provides nutrients and oxygen to third and innermost layer, the retina, a tissue formed by a network of nerve and glial cells [1, 2].

1.1.1. The retina

In all vertebrates, the retina has an organizational pattern of 10 layers of body cells, nerve plexuses, limiting membranes, pigment epithelium, and nerve fibers (**Figure 1**). This laminar tissue, responsible for capturing and initiating the processing of luminous information for image formation, has a complex organization, with five main types of neurons: photoreceptors, bipolar cells, horizontal cells, amacrine cells, and ganglion cells. The neuroanatomist Santiago Ramón y Cajal was the first to describe, in 1893 [1], this thin neural tissue, with a 10-layered division.

The retinal pigment epithelium is the outermost layer, formed by epithelial cells with pigment granules, and has a number of metabolic functions essential for retinal homeostasis and activity, such as nutrients and oxygen supply, and cycling of the photosensitive chromophore (retinal) [3–6]. The photoreceptor layer (PL) is formed by the outer and inner segments of these

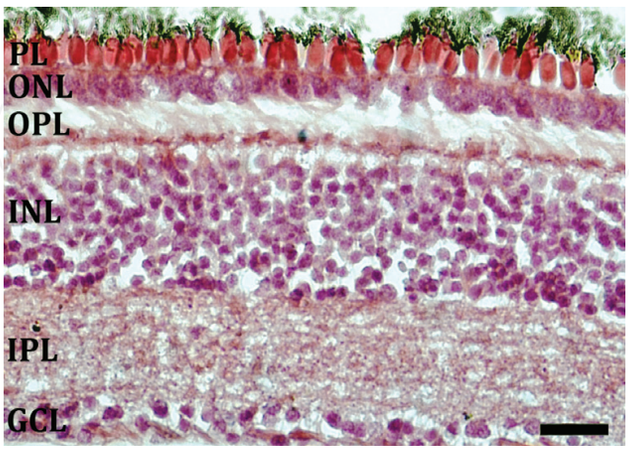

Figure 1. Photomicrograph of a cross section of the diurnal snake, *Tomodon dorsatus*, labeled with hematoxylin and eosin. PL, photoreceptor layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 20 μm.

neurons (cones and rods), specialized in capturing and converting the light energy into electrochemical energy and transmitting this information to the cells of the following layer. The outer limiting membrane (OLM), located below the PL, is formed by the extensions of Müller cells (glial cells) and is followed by the outer nuclear layer (ONL), with the photoreceptors nuclei. These first-order neurons make synaptic contact with second order neurons, bipolar and horizontal cells, in the outer plexiform layer (OPL). Bipolar, horizontal, and amacrine cell bodies are located in the inner nuclear layer (INL), and these cells make synaptic contact with the ganglion cells (third order neurons), in the inner plexiform layer (IPL). The cell bodies of ganglion cells and displaced amacrine cells form the ganglion cell layer (GCC). The ganglion cell axons form the nerve fiber layer (NFL) and come together to form the optic nerve, which conducts information from the retina to the higher visual centers in the brain. The inner limiting membrane (ILM) is also composed of laterally contacting extensions of Müller cells [1].

The photoreceptors contain visual photopigments, which are responsible for capturing luminous information and initiating visual processing. Two main types of photoreceptors are usually present in vertebrate retinas, cones, and rods. The outer segments of these cells consist of stacked membranous disks containing the visual photopigments. The latter are formed by a membrane protein, opsin or rhodopsin, coupled to a chromophore, responsible for the absorption of photons and the beginning of the visual processing [7, 8]. The higher number of photopigments in rods provides greater absorption capacity of photons, which makes these cells more sensitive to light compared to cones. Rods are responsible for the scotopic (nocturnal) vision system, which is highly sensitive, with a large capacity of light capturing and signal amplification generated by a single photoizomerization event and the great synaptic convergence, with many rods attached to one ganglion cell, through the bipolar cells, but with a low visual acuity, due to the high degree of convergence. The photopic (diurnal) visual system mediated by cones has less sensitivity but greater visual acuity [7–9]. Under high luminous intensity, rods are saturated, while cones are activated. During the night, rods are activated with the illumination below the activation threshold of cones [10]. Nocturnal animals usually have retinas with predominance of rods, whereas diurnal animals possess greater amount of cones. Different types of photopigments capture maximally photons with different wavelengths. The presence of distinct photoreceptors in the retina, with different opsin types, together with a postreceptor mechanism capable of comparing the signal transmitted by these neurons, is the first step to enable the color vision [11].

More recently, a third photoreceptor class was described in the inner retina of many vertebrates [12–14]. The melanopsin-containing ganglion cells, known as intrinsically photosensitive retinal ganglion cells (ipRGCs), are activated directly by light. These cells give rise to circuits that process important physiological functions, such as the circadian rhythm synchronization and pupillary light reflex [15–17]. They constitute the nonimage forming visual system. The ipRGCs represent about 1–3% of the retinal ganglion cells in mammals [14, 18]. In other vertebrates, as fish [19, 20] and birds [21, 22], melanopsin-containing neurons were described not only in the GCL but also in the other retinal layers.

The ganglion cells are on average larger than the other retinal neurons and have myelinated axons, with large diameters, capable of transmitting the electrical messages of the visual signal

generated by the photoreceptors and processed in the inner retina [7], to the receptive areas of the brain, many millimeters or centimeters away from the retina. Their density and topographic distribution in the retina are important factors in determining the upper limits for the spatial resolution power of the eye [23–26].

In short, the highly complex and standardized laminar pattern of the retina is observed in all vertebrates. However, remarkable differences related to the specific cell types, and their density and distribution in the retina, the so-called retinal specializations, are observed among the different species and are related to specific habitats, behaviors, and the species' visual ecology.

1.1.2. Retinal specializations

A higher concentration of retinal neurons is observed in regions of greater demand for a good image quality [24, 25, 27–34]. Some studies have shown that cell distribution correlates better with species behavior and habitat than with phylogeny, and that phylogenetically related species may have different patterns of distribution and organization of the neural elements and vice versa [28–30, 35, 36]. The retinal specializations are areas of higher cell density compared to neighboring areas and include visual streaks, *area centralis*, and fovea [2, 32, 37, 38]. Visual streaks are elongated regions of higher cell density and can be horizontal or vertical. The horizontal streak is common in vertebrates that occupy habitats whose visual field is dominated by the horizon, such as the air-land interface of terrestrial species or water-land interface of aquatic species [32] (**Figure 2**). The horizontal streak provides a panoramic view of the environment without the need for a high degree of eye movement [28, 32]. Examples of horizontal streaks are observed, for instance, in retinas of the turtle *Trachemys scripta elegans* [39, 40], alpacas *Vicugna pacos* [41], and the agouti *Dasyprocta aguti* [30, 42]. The vertical streak

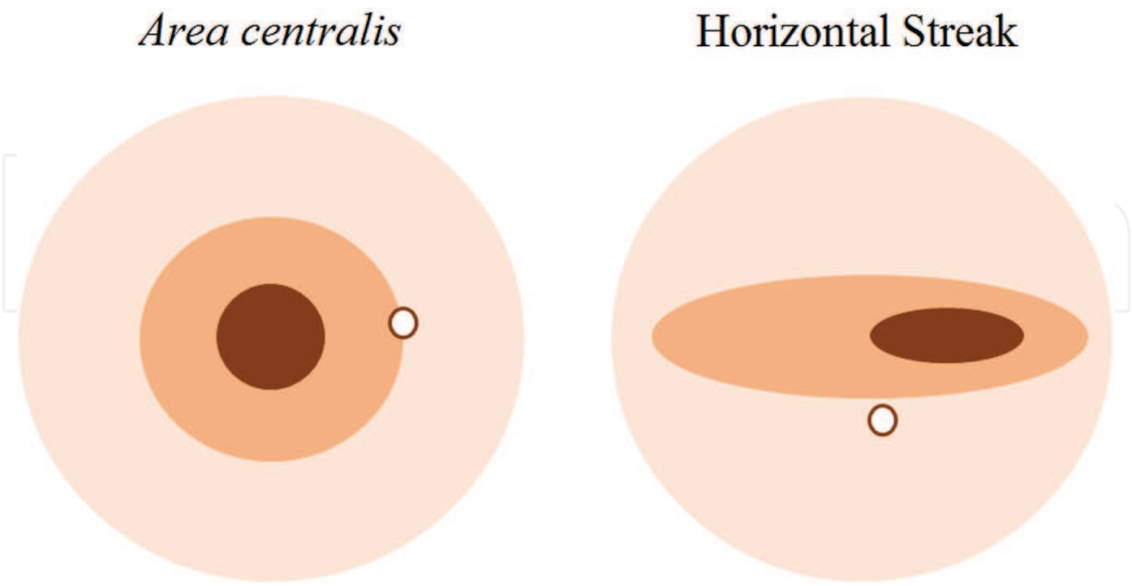

Figure 2. Representation of an *area centralis* located in the central retina, and a horizontal streak extended along the retinal meridian axis. The variation in cell density is represented by the color gradient, where the darker color indicates the region of higher density. The white spots represent the optic nerve head.

is also defined as an elongated increase in cell density but extends along the dorso-ventral axis of the retina and is usually located in the temporal region. This specialization is more unusual and can be observed in retinas of species whose visual field is dominated by a vertically oriented feature, such as the tree branches seen by the sloth *Choloepus didactylus* [43], the trunk of the African elephant *Loxodonta africana* [44], or the water column, seen by vertically migrating species, as the teleost fish *Howella sherborni* [45].

Many species have a circular area of higher cell density called *area centralis* (**Figure 2**). This type of specialization does not have the advantage of perceiving the panoramic visual field like that provided by the horizontal streak and requires a greater degree of eye movement to locate prey and the presence of potential predators [28, 32]. The *area centralis* is usually located in the temporal region of the retina, thus included in the frontal binocular visual field, which is also determined by the position of the eyes on the head. In some species, there is a concentric arrangement of the isodensity contours within the *area centralis*, while in others, the cellular arrangement is described as anisotropic, because the isodensity contours are not concentric and the density distribution is irregular [32, 38].

According to Hughes' "terrain theory" [28], terrestrial animals inhabiting open fields generally have a horizontal streak with high density of photoreceptors and ganglion cells. Since the streak provides a panoramic view of the environment, there is no need for eye movements for detection of objects along the horizon line, an appropriate feature for the field extension vision and perception of the approach of predators. Arboreal species or those from dense forests generally have an *area centralis*, with a higher density of cones and ganglion cells, which confers better acuity to this circular region. The specific function of the *area centralis* may vary depending on the predominant type of retinal ganglion cell [38, 46]. About 20 different types of ganglion cells have been described in the retina of mammals [47, 48] and can be distinguished based on their morphology, stratification pattern, and specific functions.

Some primates, reptiles, and birds have a fovea, a specialization of the *area centralis*, characterized by the lateral displacement of cells from the inner layers of the retina and an increase in the density of ganglion cells in the perifoveal region [2, 32, 49]. In the fovea, there is an increase in the density of photoreceptors with more elongated outer segments and smaller diameter, and the presence of only cones favors greater acuity in this region [2, 37, 39, 50]. There are two main types of foveae—the convexiclivate fovea, with a steep slope on both sides of the depression, as observed in some fish and birds, or the concaviclivate fovea with a shallow depression, as in monkeys and humans [32]. In some species, as the sacred kingfisher bird, *Halcyon sancta*, the presence of two foveae has been described; a temporal that acts in the binocular vision and a nasal involved in monocular vision [51].

1.1.3. Spatial resolving power

Variations in the visual acuity may reflect ecological differences among species and are limited by the diffraction and optical aberration characteristics of the eye, the density of photoreceptors and ganglion cells, and by variables such as refraction error, ambient illumination, and contrast [52]. Lisney and Collin [26] analyzed the retinas of several species of elasmobranchs (sharks and rays) and observed that species with lower resolution power tend to be relatively

less active and feed on benthic invertebrates and small fish, while more active, predatory species that usually feed on larger prey have a greater eye resolving power.

The visual acuity of an animal can be measured using different approaches, such as behavioral tests, response to a stimulus, ocular movements (preferential look), electrophysiological recording, or it can be estimated from anatomical data [41]. Because the ganglion cells constitute the final output of visual information from the retina to the higher visual centers, their density represents a limiting factor of the spatial resolution power of the eye and the ability of the animal to distinguish fine details of the objects [24]. Thus, the peak density of ganglion cells in combination with the eye focal length may be used to infer the maximum spatial resolution power of an animal [24, 25, 33, 34, 53]. These estimated values are usually very close to the acuity values obtained from more direct methods for many species in which both measurements were compared [54–58].

The retinal specializations and the spatial resolution power of the eye are closely associated with the animals' visual ecology. Studies on these aspects of the visual system, which include the analysis of the density and distribution of retinal neurons and the specific area of higher degree of visual acuity, bring valuable information on the species biology and are often more related to ecological and behavioral features than to phylogeny. In order to better understand the evolution and functioning of this complex sensory system, it is of great value to compare closely related species with ecological differences. An excellent model for this type of comparative study is the group of snakes, given their great diversity and the variety of ecological niches occupied by phylogenetically close species.

1.2. Snakes: characteristics of the group and adaptations of the visual system

The infraorder Serpentes is characterized by body stretching, absence of limbs, eyelids and external ears, and the presence of forked tongue [59] and is subdivided into two main groups. The Scolecophidia group (blind snakes) is composed by small fossorial snakes with reduced eyes that feed on small prey as termites and ants. The Alethinophidia group is composed by a greater diversity of species, with two major groups, the paraphyletic Henophidia group, with about 180 species, including pythons and boas, and the Caenophidia group, with about 2500 species [60, 61]. Snakes from the Caenophidia group are found in virtually every portion of the biosphere, except for the poles, some islands, and the ocean deep [62]. The great diversity of this group, with species adapted to a great variety of habitats, can be explained by the occurrence of a number of adaptations that favored their dispersion [63–65] and the specialization of their sensory systems that evolved to allow their survival and adaptive radiation. The Caenophidia group is therefore characterized by a great diversity of species, with differences in the circadian activity patterns and in the habitats occupied, including terrestrial, arboreal, cryptozoic or fossorial, as well as aquatic environments, which include marine or fresh water habitats [66].

Despite the great diversity of snake' species and ecological and behavioral features, very few studies have investigated their retinal specializations. To date, only three studies described the distribution of neurons in snakes' retinas. Wong [67] described a visual streak for cones and

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GCL cells in retinas of the terrestrial *Thamnophis sirtalis*. The arboreal *Philodryas olfersii* has a horizontal streak and two discrete anisotropic *area centralis* in the central and temporal regions of the retina, while the closely related species, the terrestrial *P. patagoniensis*, has an anisotropic *area centralis* in the ventro-nasal retina [36]. In retinas of marine snakes from the Hydrophiidae family, Hart et al. [34] observed a horizontal streak in *Lapemis curtus*, *Aipysurus laevis*, and *Disteira major*, with discrete *area* in the temporal and nasal quadrants. The species *L. curtus* and *D. major* also have a ventral *area*.

The upper limits of spatial resolving power, estimated based on the ganglion cell peak density and the eye focal length, varied between 2.3 and 2.8 cpg in diurnal and terrestrial snakes [36, 67] and were lower in marine species, ranging between 1.1 and 2.3 cpd [34]. The lower values of marine snakes were attributed to reduced eye size and differences in the photic properties of water compared to air. A higher visual acuity, 4.9 cpd, was measured by recording evoked responses from telencephalon in the aquatic snake *Nerodia sipedon pleuralis* [68]. Compared to other reptiles, snakes had lower values of visual acuity: 6.1 cpd in the red-eared slider turtle (*Trachemys scripta elegans*) [69], 6.8 cpd in the sleepy lizard (*Tiliqua rugosa*) [70], and 13.6 cpd in the anoline lizard (*Anolis carolinensis*) [71]. Compared to mammals, snakes had higher values than the opossum *Didelphis aurita* (1.3 cpd) [72], rats (1 cpd) [73, 74], and mice (0.6 cpd) [75] but lower than cats (10 cpd) [54], and humans (60 cpd) [76].

In short, the diversity of species and the variability of habitats used by snakes point to important adaptations of their visual system. Studies on the characteristics and adaptations of the visual system of snakes are extremely scarce in view of the large number of species. Based on their ecological diversity, caenophidian snakes represent a good model for testing hypotheses of correlations between retinal specializations and behavioral ecology. Thus, we analyzed and compared the density and distribution of the GCL cells and estimated the eye spatial resolving power of 12 species from the Colubridae family, with variety regarding their daily activity pattern, and the preferential substrate: arboreal, terrestrial, fossorial, or aquatic. The analysis revealed the presence of different specialization types, visual streak or *area centralis* located in different regions of the retina, depending on the species. The population of GCL cells and the estimated upper limit of the spatial resolving power varied among diurnal and nocturnal species. The diversity of retinal specializations observed in this study seems to be related to a variety of ecological and behavioral features, pointing to the complexity of the evolution and adaptations of the retinal structure.

2. Assessing cell density and topographic distribution across the retinas and estimating the spatial resolving power

In this study, retinas of 12 Colubridae snakes, 6 considered as primarily nocturnal and 6 as primarily diurnal (**Table 1**), were collected and dissected for wholemount and Nissl-staining technique. The adult specimens were obtained at the Butantan Institute, São Paulo, Brazil, and were euthanized with a lethal injection of sodium thiopental (thiobarbiturate ethyl sodium, 30 mg/kg). Following euthanasia, the eyes were enucleated, and the axial length was

Family	Subfamily	Tribe	Species	Biome	Habitat	Substrate	Activity pattern
Colubridae	Colubrinae		*Chironius bicarinatus*	AF	F	Ar/Te	D
			Spillotes pullatus	AF	F	Ar/Te	D
	Dipsadinae		*Atractus pantostictus*	CE/AF	F	Su	N
			Atractus reticulatus	CE/AF	F	Su	N
			Dipsas albifrons	AF	F	Ar/Te	N
			Sibynomorphus mikanii	CE/AF	F, O	Te	N
			Sibynomorphus neuwiedi	AF	F	Ar/Te	N
			Erythrolamprus aesculapii	CE/AF	F, O	Te	D
		Xenodontini	*Erythrolamprus miliaris*	AF	F	Aq/Te	D
			Erythrolamprus poecilogyrus	CE	F, O	Te	D
		Pseudoboini	*Oxyrhopus guibei*	CE	F, O	Te	N
		Tachymenini	*Tomodon dorsatus*	AF	F	Te	D

CE, Cerrado; AF, Atlantic Rain Forest; O, open area; F, forested area; Ar, arboreal; Te, terrestrial; Su, subterranean; Aq, aquatic; D, primarily diurnal; N, primarily nocturnal. Biome, habitat and substrate were determined based on [78]. Activity pattern was stablished based on [77, 79].

Table 1. Species, habitat and daily activity pattern.

measured. The cornea, ciliary body, and lens were removed, and the lens diameters were measured. A small radial incision was made in the dorsal region of each eyecup, for retinal orientation. The retinas were dissected from the eyecup, the pigment epithelium was separated, and the vitreous humor was removed. The retinas were fixed in 10% formalin. After these procedures, the specimens were fixed in 10% formaldehyde and preserved in the herpetological collection of the Butantan Institute. The animal procedures were done in accordance with the ethical principles of animal management and experimentation established by the Brazilian Animal Experiment College (COBEA). Species daily activity pattern and ecological features were established based on [77–79] (**Table 1**).

The retinas were flattened on gelatinized slides, with the GCL side facing up. Small radial incisions were made to allow the retinas to flatten and adhere to the slide. To label the retinal ganglion cells, we used Nissl-Staining procedures as described previously [36]. Glial cells were identified by their dark staining, small size, and rounded profile [34, 67, 83] and were not included in the counts. However, ganglion cells and displaced amacrine cells could not be reliably differentiated from each other, and both were included in the GCL cell counting [36, 80–85]. To analyze the density and distribution of GCL cells in wholemount retinas, we used a systematic random sampling and the fractionator principle [53, 82, 86–88]. The coordinates of the retinal edges were plotted on an Excel spreadsheet, and cells were counted at regular intervals defined by a sampling grid, ranging from 220 × 220 μm up to 680 × 680 μm, depending on the size of each retina (**Table 2**). The coordinates of the sampled fields were plotted on the same Excel spreadsheet, as well as the number of cells counted per field. The counting was performed directly under a Leica DMRXE microscope with a 100× oil objective

Species	Counting Frame (μm × μm)	Grid (μm × μm)	Area sampling fraction	Number of sites counted
Diurnal				
C.bicarinatus	74 × 74	620 × 620	0.014	154
E. aesculapi	74 × 74	520 × 520	0.020	125
E. miliaris	74 × 74	320 × 320	0.054	217
E. poecilogyrus	74 × 74	320 × 320	0.054	155
S. pullatus	74 × 74	680 × 680	0.010	190
T. dorsatus	74 × 74	320 × 320	0.050	184
Nocturnal				
A. pantostictus	74 × 74	230 × 230	0.100	55
A. reticulatus	74 × 74	220 × 220	0.110	56
D. petersi	74 × 74	340 × 340	0.048	160
O. guibei	74 × 74	310 × 310	0.057	117
S. mikanii	74 × 74	320 × 320	0.053	133
S. neuwiedii	74 × 74	220 × 220	0.115	272

Table 2. Stereological parameters used to estimate the number and distribution of retinal ganglion cell layer (GCL) cells in retinas of colubrid snakes, using the optical fractionator method.

(numerical aperture, NA = 1.25), equipped with a Nikon Digital Sight DS-U3 DSRi1 camera and the software NIS-Elements AR Microscope Imaging (Nikon Instruments, Melville, NY, USA). A counting frame at 74 × 74 μm was imposed on each sampled frame (**Table 2**). Cells were counted when inserted fully inside the counting frame or if touched the acceptance lines, without touching the rejection lines [86]. The number of cells quantified at each sampled field was entered in the Excel spreadsheet and converted into density value of cells per mm^2, by dividing the number of cells by the frame sampling area. The total number of GCL cells was estimated by multiplying the total number of cells counted by the inverse of the area sampling fraction (asf). The asf is calculated dividing the area of the counting frame by the area of the sampling grid, according to the algorithm: N total = ΣQ × 1/asf, where ΣQ is the number of counted cells [53, 89, 90] (**Table 2**). The average cell density of each retina was estimated from the average density values of each sampled field. The coefficients of error (CE) were calculated using the method proposed by Scheaffer et al. [91] and were <0.02 for all retinas, indicating that the total cell number estimates had a high degree of accuracy [53, 87, 92].

The coordinates of each sampled frame and the cell density values were used to elaborate the topographic maps, with the software OriginPro 8.1 (Northampton, MA, USA). The position of the retina was determined based on the radial incision made in the dorsal region during the dissection procedures and based on the optic nerve located in the ventral and temporal retinal quadrant in snakes. The reconstructed images were processed using the software Adobe Photoshop CS3 (Adobe Systems, Inc.).

We estimated the theoretical upper limits of the spatial resolving power based on the peak density of presumed ganglion cells and the estimated focal length of the eye. The focal length of the eye is represented by the posterior nodal distance (PND), which corresponds to the distance from the lens center to the choroid-retina border [93, 94]. In a broad analysis of different vertebrate species, Pettigrew and colleagues [24] proposed that the PND of diurnal vertebrates has a mean of 0.67 of the eye's axial length and that of nocturnal vertebrates has a mean of 0.52 of the eye's axial length. However, Hauzman and colleagues [36] estimated a focal length of 0.52 for diurnal colubrids. In this study, we accessed the eye's focal length of two colubrids, the diurnal *Tomodon dorsatus* and the nocturnal *Sibynomorphus neuwiedi* (**Figure 3**). For the other species analyzed, the PND was inferred from the measured axial length of the eye and the PND values obtained from the nocturnal and the diurnal species. To assess the focal length, we used the method described by Lisney and Collin [26], and for both species, the estimated focal length was 0.52 of the eye's focal length (**Figure 3**).

To estimate the theoretical peak of spatial resolving power, we applied the method proposed by Hart [85], wherein the distance **d** subtended by one degree on the retina is determined from the PND and calculated according to the formula: $\mathbf{d} = (2\pi PND)/360$. Assuming that the spacing between the ganglion cells is the limiting factor of the spatial resolving power, we applied two different approaches, one in which we assume that the retinal ganglion cell fields are arranged in approximately a hexagonal array and the other in which we assume that the ganglion cell fields are arranged in an approximately square lattice. For both approaches, we estimated the average spacing between the cells using the value of peak density of GCL cells. Assuming a hexagonal array, the average spacing between the cells (S) was estimated

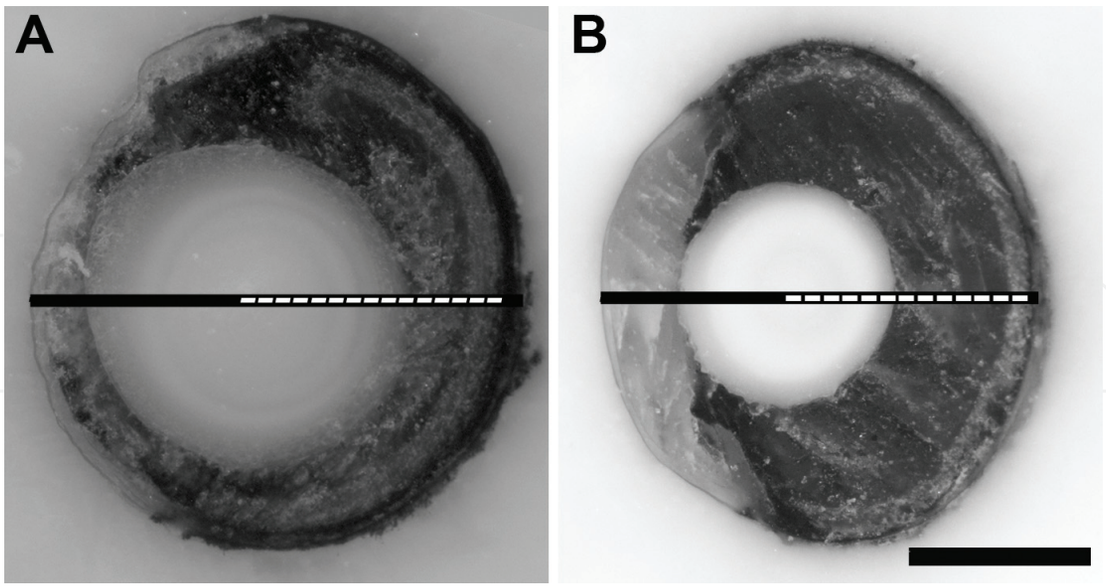

Figure 3. Transversal section of the eye of the nocturnal snake *S. neuwiedi* (A) and the diurnal *T. dorsatus* (B). The posterior nodal distance (PND) from the lens center to the edge of the retina-choroid is represented by the white dashed line, and correspond to about 52% of the axial length of the eye. Scale bar 1 mm.

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according to the formula: S2 = 2/(D√3), where D is the peak density of GCL cells in mm^2. In the second approach, the linear density (cells/mm) was estimated by calculating the square root of D (cells mm^{-2}), and then was divided by 2, because at least two cells are required to detect 1 cycle of a given spatial frequency [90]. The maximum spatial (Nyquist) frequency (v) of a sinusoidal grating resolvable by these cell arrangements [95] was calculated using the formula $v = 1/(S\sqrt{3})$. This value was multiplied by the distance **d** to obtain the value of spatial resolution in cycles per degree [24, 90].

Statistical analyses were performed using the program SPSS v.20.0 Statistic (IBM Corporation, Armonk, NY, USA), to compare the population of GCL cells and the estimated upper limit of the spatial resolving power in diurnal and nocturnal colubrids, using the parametric t test and the nonparametric Mann-Whitney test. All data were log 10 transformed prior to analysis. The distribution of values for each variable in each group was evaluated by the Kolmogorov-Smirnov test, and the homoscedasticity between the groups was assessed by the Levene test. The t test for independent samples was performed to verify possible differences between the mean of the groups, for each variable analyzed. There was no disagreement in terms of statistical significance between the Mann-Whitney tests performed, which reinforces the results of the t tests. The level of significance for all comparisons was 5%.

In the retinal wholemounts, we were able to differentiate the neuron population (ganglion cells and displaced amacrine cells) from the nonneuron cell population (glial cells) (**Figure 4**). Diurnal and nocturnal colubrid snakes differed statistically in the total population of GCL cells and the estimated spatial resolution but not in the mean density of GCL cells. The average cell

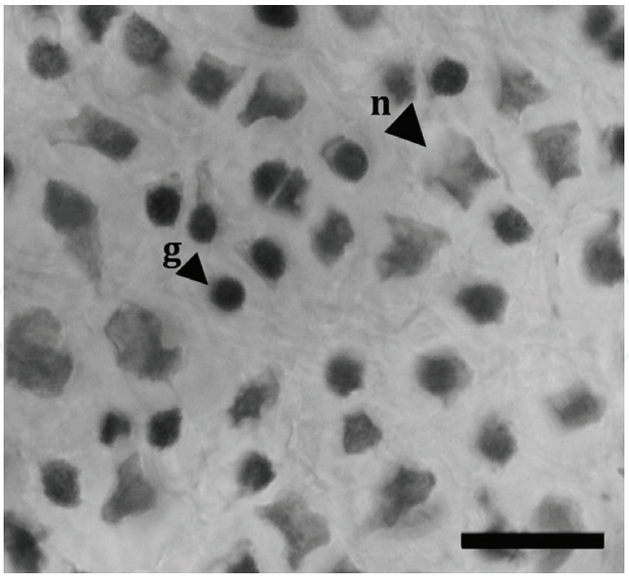

Figure 4. Photomicrograph of the Nissl-stained retinal GCL of the diurnal snake *T. dorsatus*. n, neurons; g, glia cells. The digital image was processed using adobe Photoshop CS3 for scaling, resolution, and adjustment of the levels of brightness and contrast. Scale bar = 10 μm.

Species	Retinal area (mm^2)	Total number of cells	CE	Mean cell density (cells/mm^2)	Eye axial length (mm)	Peak density of GCL cells (cells/mm^2)	Visual acuity (cpd)*	Visual acuity (cpd)**
Diurnal								
C. bicarinatus	59	394,096	0.01	6721 ± 1634	5.5	11,623	2.9	2.7
E. aesculapii	33	247,386	0.01	7474 ± 2308	4.5	12,176	2.4	2.3
E. miliaris	22	172,525	0.01	7842 ± 2034	4.0	12,281	2.2	2.0
E. poecilogyrus	16	110,153	0.01	7016 ± 2258	3.0	13,381	1.7	1.6
S. pullatus	88	614,553	0.01	7025 ± 1355	7.0	10,815	3.5	3.3
T. dorsatus	19	195,131	0.01	10,297 ± 1934	4.0	14,759	2.4	2.2
Mean ± SD	39 ± 28	288,974 ± 186,079		7729 ± 1318	4.7 ± 1.4	12,506 ± 1389	2.5 ± 0.6	2.3 ± 0.6
Nocturnal								
A pantostictus	3	31,404	0.02	11,019 ± 2410	1.3	16,788	0.8	0.8
A reticulatus	3	23,007	0.02	8490 ± 2177	1.8	11,992	1.0	0.8
D. albifrons	18	97,524	0.02	5341 ± 1189	3.5	7933	1.5	1.4
O. guibei	11	59,285	0.02	5327 ± 1140	2.9	8798	1.3	1.2
S. mikanii	14	58,621	0.02	4270 ± 1175	3.9	7195	1.6	1.5
S. neuwiedii	13	77,298	0.01	5987 ± 1200	3.7	9531	1.8	1.6
Mean ± SD	10 ± 6	57,856 ± 27,815		6739 ± 2530	2.9 ± 1.1	10,373 ± 3550	1.3 ± 0.4	1.2 ± 0.4

CE, Schaeffer coefficient of error; PND, posterior nodal distance; cpd, cycles per degree; SD, standard deviation of the mean.
*Estimated visual acuity assuming a hexagonal array.
**Estimated visual acuity assuming a square array.

Table 3. Stereological analysis of the population of cells in the retinal ganglion cell layer (GCL) of colubrid snakes, and the anatomical parameters used to estimate the upper limit of spatial resolution.

population in the GCL was 57.856 ± 27.815 cells in the retinas of nocturnal snakes and 288.974 ± 186.079 cells in retinas of diurnal snakes (t(10) = 4.7, p = 0.001) (**Table 3** and **Figure 5**). The mean cell density was 6.739 ± 2.530 cells/mm^2 in nocturnal species and 7.729 ± 1.318 cells/mm^2 in diurnal species (t(10) = 1.2, p = 0.28) (**Table 3** and **Figure 5**). The mean spatial resolution assuming a hexagonal arrangement was 1.3 ± 0.4 cpd in nocturnal snakes and 2.5 ± 0.6 cpd in diurnal snakes (t(10) = 3.9, p = 0.003) (**Table 3** and **Figure 5**). Similar values were obtained for the assumption of a square lattice: 1.2 ± 0.4 cpd and 2.3 ± 0.6 cpd, in nocturnal and diurnal species, respectively (**Table 3**).

The ganglion cell isodensity contour maps showed different types of retinal specializations, which may be related to species daily activity pattern and differences in habitat preferentially used (**Figure 6**). Poorly defined horizontal streaks with higher cell densities in the temporal

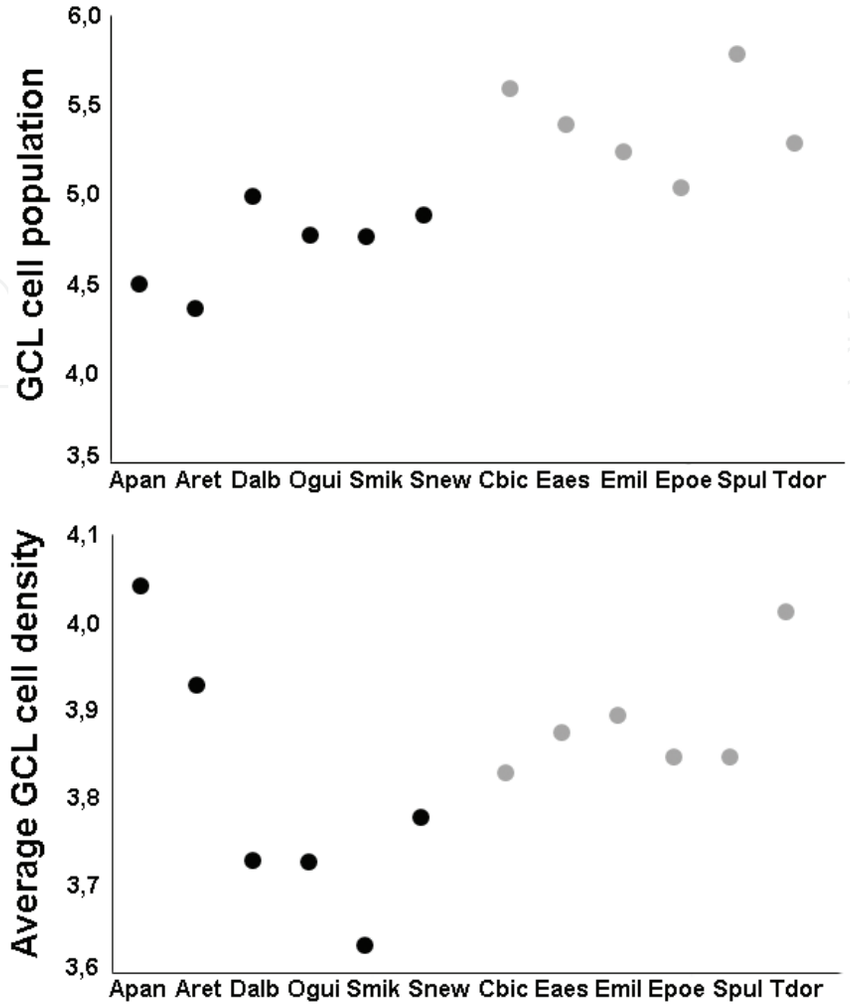

Figure 5. Total number (upper plot) and mean density (lower plot) of GCL cells in nocturnal (black) and diurnal (gray) colubrid snakes. All of the data were $\log_{10}$ transformed. Apan, *A. pantostictus*; Aret, *A. reticulatus*; Dalb, *D. albifrons*; Ogui, *O. guibei*; Smik, *S. mikanii*; Snew, *S. neuwiedii*; Cbic, *C. bicarinatus*; Eaes, *E. aesculapii*; Emil, *E. miliaris*; Epoe, *E. poecilogyrus*; Spul, *S. pullatus*; Tdor, *T. dorsatus*.

region were observed in diurnal species that inhabit distinct habitats: the arboreal species *C. bicarinatus* and *S. pullatus*, the terrestrial species *E. aesculapii* and *E. poecilogyrus*, and the semiaquatic species *E. miliaris*. In the terrestrial snake *T. dorsatus*, we observed a scattered distribution with anisotropic *area centralis* in the ventro-temporal retina. Among the nocturnal species, we observed anisotropic *area centralis* located in the dorso-temporal retina of the fossorial species *A. pantostictus* and *A. reticulatus*. An anisotropic *area centralis* in the temporal retina was observed in the terrestrial and nocturnal snake *O. guibei*, and a scattered GCL cell distribution was observed in the other three nocturnal species: the semiarboreal snakes *D. albifrons* and *S. neuwiedi*, with higher density in the ventral retina, and the terrestrial snake *S. mikanii*, with higher density in the central retina. Examples of horizontal streaks, anisotropic *area*, and scattered and nondefined distributions are shown in **Figure 6**.

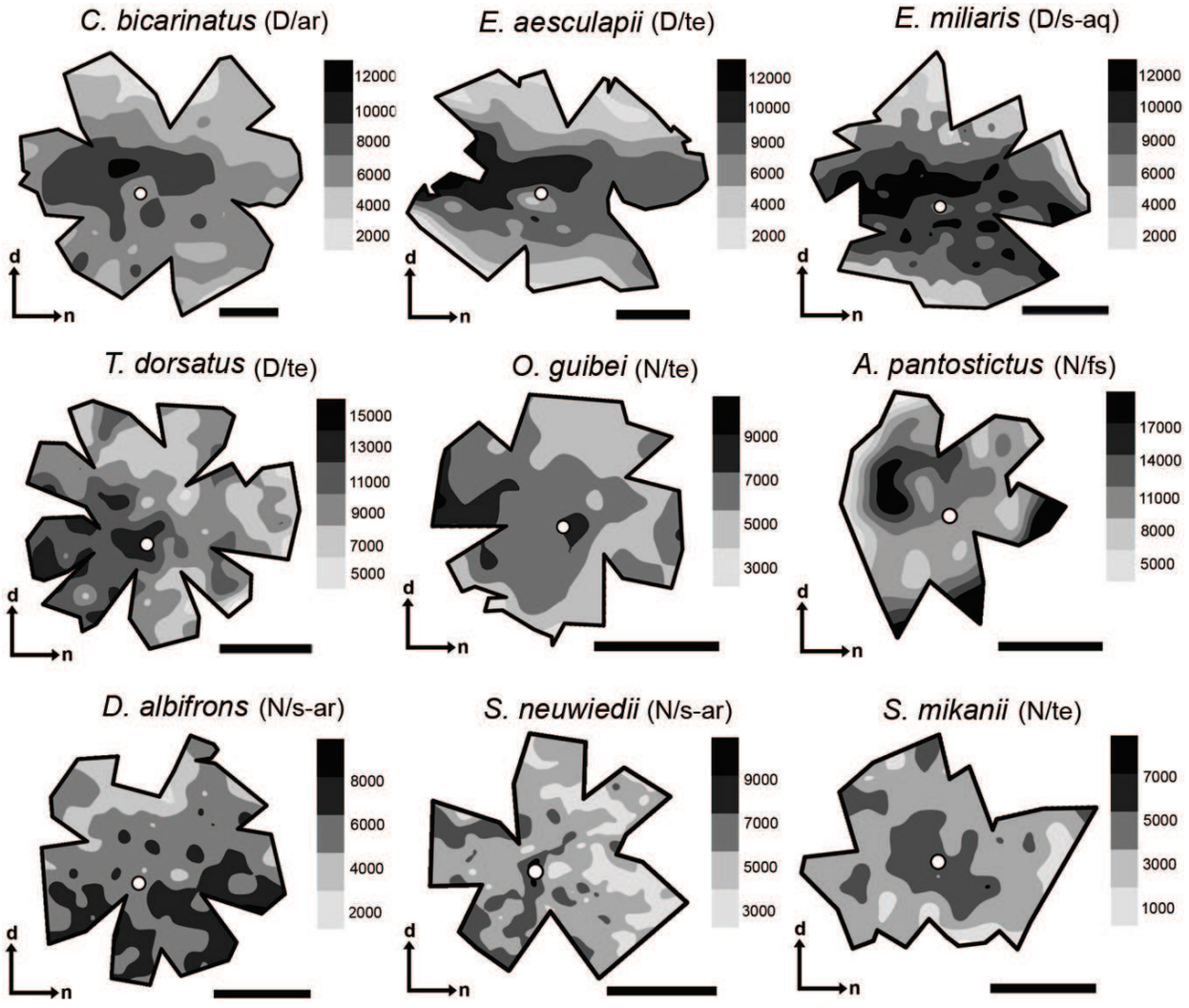

Figure 6. Topographic maps showing the distribution of cells in the GCL in retina of diurnal and nocturnal Colubrid snakes. The upper three maps show poorly defined horizontal streaks in diurnal species. The middle maps show anisotropic *area centralis* in diurnal and nocturnal species and the lower three maps show scattered and nondefined distributions in retinas of nocturnal snakes. The daily activity pattern and species habitat are indicated next to the name of each species, in the order (activity pattern/habitat): D, primarily diurnal; N, primarily nocturnal; ar, arboreal; fs, fossorial; s-ar, semiarboreal; s-aq, semiaquatic; te, terrestrial. The gray gradient bars represent the number of cells per mm^2. The position of the optic nerve head is depicted as a white circle. d = dorsal; n = nasal. Scale bars = 2 mm, except for C. bicarinatus and E. aesculapii, where scale bar = 1 mm.

3. Discussion

3.1. Density of GCL cells and the estimated spatial resolving power

To our knowledge, this is the first study to evaluate and describe the density and distribution of neurons in retinas of nocturnal Colubridae snakes. The average of the total population of GCL cells was significantly lower in nocturnal species (57,856 ± 27,815 cells) compared to diurnal species (288,974 ± 186,079 cells). No significant difference of the mean density of GCL

cells was observed between diurnal and nocturnal species, although this may reflect the low species sampling, with a high sampling variability (**Figure 5**). Among the nocturnal snakes, the fossorial *A. reticulatus* had the lowest value of GCL cell population (23,007 cells), while the highest was seen in the semiarboreal *D. albifrons* (97,524 cells). Among diurnal snakes, the semiaquatic *E. miliaris* had the lowest value (172,525 cells) and the arboreal *S. pullatus* (614,553 cells) the highest.

Data from the literature show similar density values for diurnal and terrestrial snakes. The population of GCL cells in the semiarboreal *P. olfersii* was 307,605 ± 80,422 cells, in the terrestrial *P. patagoniensis* 350,294 ± 64,756 cells and in the terrestrial *T. sirtalis* 209,800 ± 1150 cells [36, 67]. The estimated densities in diurnal marine snakes were lower than those observed for diurnal terrestrial species (104,011 cells in *A. laevis*, 31,977 cells in *D. major*, and 72,597 cells in *L. curtus*) [34] and were similar to the values estimated for the nocturnal colubrids.

The upper limits of the spatial resolving power were also significantly higher in diurnal snakes (2.5 ± 0.6 cpd), which points to the importance of a better image quality in snakes with diurnal habits that actively forage during photoperiods of higher illumination. Similar values were reported for other terrestrial and diurnal species: 2.6 cpd in *P. olfersii*, 2.7 cpd in *P. patagoniensis*, and 2.3 cpd in *T. sirtalis* [34, 36, 67]. The average spatial resolving power in nocturnal colubrids was 1.3 ± 0.4 cpd, which was similar to the values estimated for the marine snakes *D. major* and *L. curtus*: 1.1 cpd and 1.6 cpd, respectively [34]. The marine *A. laevis* had a higher estimated visual acuity (2.3 cpd), which was associated to its crevice-foraging hunting strategies [34]. The lower values estimated for marine and for primarily nocturnal terrestrial species may be associated to the smaller eye's axial diameter and shorter focal length. In nocturnal snakes, visual acuity may be compensated by light sensitivity.

Morphological studies revealed that diurnal snakes from the Caenophidia group, which include the Colubridae and Hydrophiidae families, have pure cone retinas, with no typical rod-like photoreceptor [2, 34, 36, 67, 96–98], and a lower photoreceptor density, compared to nocturnal species [36, 98]. The presence of only cones in retinas of diurnal snakes should contribute to higher spatial resolution [99], given the lower convergence from cones to ganglion cells.

These important differences in the retinal morphology and the upper limits of the spatial resolving power between diurnal and nocturnal snakes and between aquatic and terrestrial snakes indicate how the variety of environments and circadian activity patterns plays a role in the adaptation of the visual system and influence essential aspects of vision.

3.2. Distribution of neurons in the retina and visual ecology of snakes

This comparative study on the distribution of GCL neurons in retinas of Colubridae snakes revealed the variety of retinal specializations, which indicates the complexity of the adaptive strategies of the snakes' visual system. In the literature, only three studies described the density and topography of neurons in snake retinas [34, 36, 67], and this is the first study to describe the distribution of neurons in retinas of nocturnal snakes. In general, the diurnal species had a poorly defined visual streak extending along the meridional axis of the retina

with peak density of cells in the temporal region, while nocturnal species had an anisotropic *area centralis* in different regions of the retina, or a scattered and nondefined distribution, depending on the species (**Table 4**).

According to the terrain theory proposed by Hughes [28], species that inhabits open areas where the visual field is dominated by the horizon should preferably have a horizontal streak, which favors the panoramic view of the environment, without the constant need for eye or head movements. On the other hand, animals that live in forested areas, where the visual field is obstructed by foliage and should have an *area centralis*, which favors the spatial resolution of this circular area. However, the distribution pattern observed in snakes' retinas does not support this theory. For instance, visual streaks were observed in arboreal snakes that inhabit preferentially forested areas, such as *P. olfersii* [36], *C. bicarinatus* and *S. pullatus*, and on the other hand, *area centralis* were observed in species that inhabit preferentially open areas, such as *P. patagoniensis* [36] and *O. guibei*.

In the literature, we find some studies that showed the presence of a horizontal streak in species of mammals where the horizon is not a relevant feature of their habitat or the absence of this type of distribution in species that inhabit open fields [30, 100, 101]. Stone [102] suggested that the topography of the retina must be a phylogenetically inherited trait and does not necessarily represent an adaptive condition of a lifestyle of a given species. However, if this

Subfamily	Species	Biome	Habitat	Substrate	Activity pattern	Diet	Retinal specialization
Colubrinae	*C. bicarinatus*	AF	F	Ar/Te	D	an	HS
	S. pullatus	AF	F	Ar/Te	D	ma, av	HS
Dipsadinae	*A. pantostictus*	CE, AF	F	Su	N	ol	AC—dorsal
	A. reticulatus	CE, AF	F	Su	N	ol	AC—dorsal
	D. albifrons	AF	F	Ar/Te	N	mo	Diffuse—ventral
	S. mikanii	CE, AF	F, O	Te	N	mo	Diffuse—central
	S. neuwiedii	AF	F	Ar/Te	N	mo	Diffuse—ventral
	E. aesculapii	CE, AF	F, O	Te	D	sn	HS
	E. miliaris	AF	F	Aq/Te	D	an, fi	HS
	E. poecilogyrus	CE	F, O	Te	D	an	HS
	O. guibei	CE	F, O	Te	N	ma, li	AC—temporal
	T. dorsatus	AF	F	Te	D	mo	AC—ventral
	P. olfersii	CE, AF	F	Ar/Te	D	an, ma	HS*
	P. patagoniensis	CE, AF	O	Te	D	an, ma	AC—ventral*

AF, Atlantic Rain Forest; CE, Cerrado; F, forested area; O, open area; Ar, arboreal; Te, terrestrial; Su, subterranean; Aq, aquatic; D, primarily diurnal; N, primarily nocturnal; an, anurans; ma, mammals; av., birds; ol, annelids; mo, mollusks; sn, snakes; fi, fish; li, lizards; HS, horizontal streak; AC, *area centralis*. Ecological features (biome, habitat, substrate, activity patter and diet) were stablished based on [77–79].
*Data from Hauzman et al. [36].

Table 4. Snake species, ecological features and retinal specializations.

proposition was applied to snakes, one would expect to observe the same pattern of cell distribution in the retinas of the phylogenetically close-related species *P. olfersii* and *P. patagoniensis* [36], for example, or in the nocturnal Dipsadinae and close-related species *S. mikanii*, *S. neuwiedi* and *D. albifrons*, which was not the case.

Based on the results obtained for *Philodryas* retinas, Hauzman et al. [36] suggested that the microhabitat may have a more important role in the visual ecology of snakes than the habitat. The terrestrial species *P. patagoniensis*, for example, although it occupies open areas, has its visual field obstructed by foliage. Thus, an *area centralis* in the ventral region of the retina (**Table 4**) favors the spatial resolution of the superior visual field, which allows the view of aerial predators, an important defense mechanism for crawling animals [36]. The same was suggested for sea snakes with an *area centralis* in the ventral retina, *L. curtus* and *D. major* [34]. In our analysis, we also observed a ventral *area* in the retina of the primarily diurnal and terrestrial *T. dorsatus* and a scattered distribution of cells but with higher densities in the ventral retina of the primarily nocturnal and semiarboreal snakes *D. albifrons* and *S. neuwiedi* (**Table 4**). On the other hand, the two fossorial snakes, *A. pantostictus* and *A. reticulatus*, had an *area centralis* located in the dorsal region of the retina (**Table 4**). Snakes with fossorial habits usually have adaptations related to the necessity to reduce friction and allow digging tunnels in compact soil [66]. These species tend to have smaller and compact heads, almost indistinct from the rest of the body, a reduced number of scales on the head, small eyes, and shorter tails [103–105]. The retinal specializations with an *area centralis* in the dorso-temporal region may improve the spatial resolving power of the inferior and frontal visual field, thereby helping the digging habit and the search for annelids as prey [77]. Furthermore, we also observed an *area centralis* in the central retina of the primarily diurnal snake *E. poecilogyrus*, which may improve the lateral monocular vision, and a temporal *area centralis* in the retina of the primarily nocturnal snake *O. guibei*, which may improve frontal vision (**Table 4**).

A horizontal streak was observed in different diurnal snakes that inhabit a variety of habitats and occupy different substrates: terrestrial, arboreal, or aquatic (**Table 4**). This type of retinal specialization results in the formation of a sharper image that favors the visual acuity along the naso-temporal axis, and is related to the ability of a panoramic view of the visual field, without the need for head movements [32], which would reveal the location of the snake for a possible prey or for visually oriented predators. Thus, a visual streak would be an important adaptation for locomotion and foraging in different substrates. In the literature, a visual streak was observed in the semiarboreal *P. olfersii* [36], in the terrestrial *T. sirtalis* [67], and in the sea snakes *L. curtus*, *D. major* and *A. laevis* [34]. In this study, we observed this type of retinal specialization in primarily diurnal species that occupy predominantly forested areas: the terrestrial snake *E. aesculapii*, the semiaquatic snake *E. miliaris*, and the semiarboreal species *C. bicarinatus* and *S. pullatus*. We thus suggest that this type of specialization is widely spread among snakes and may be important for foraging behavior in different substrates.

In summary, the variation of the types of retinal specialization in snakes may be related to ecological and behavioral features such as the daily activity pattern, the habitat and substrate preferentially occupied, hunting strategies, and diet. Snakes that actively forage during the day and prey on fast and visually oriented preys may benefit from a horizontal streak that

enables the screening of the environment without the constant need for eye and head movements. On the other hand, snakes are active during the night spend most of the day camouflaged resting [106] and may not have the necessity of a panoramic view of the environmental provided by a visual streak. The presence of an *area centralis* or a diffuse distribution of GCL cells may also be related to food items, in that these types of distribution are observed in snakes that feed on slow moving preys, as snails and slugs: the nocturnal species *D. albifrons*, *S. mikanii*, *S. neuwiedi*, and the diurnal snake *T. dorsatus*. Mollusks are also relatively smaller and easier to handle and ingest compared to other types of prey and that may decrease the time of exposure to potential predators during feeding [107], which may also exert distinct selective pressure on the retinal specializations.

4. Conclusion and future directions

In conclusion, this broad study reveals that the retinal topography in Colubridae snakes may have suffered influences not only from the preferential habitat, microhabitat, and substrate used by the species but also to a range of features and behaviors such as daily activity pattern, foraging strategies, and diet. We suggest that horizontal streak with higher cell density in the temporal retina is a more common feature of primarily diurnal snakes from forested areas, which feed on fast moving preys. An *area centralis* is more common in nocturnal species or those that feed on slow moving preys. We also speculate that retinal specializations may have resulted from adaptations to environments and habitats that have undergone drastic and recent changes, and many features and specific adaptations of the species visual system may not be associated with the current environment but may indicate traits of the evolutionary history of the species.

It is also important to emphasize that these anatomical and morphological analyses of the visual system should be expanded not only to a broader species sampling but should also be combined with other studies, which include electrophysiological and behavioral approaches. Electrophysiological recordings, for instance, can be performed to compare the visual acuity measured with a more direct technique, with the upper limits of the eye spatial resolving power estimated from anatomical data. In addition, behavioral tests can be designed to verify how a specific morphological feature of the retina is ultimately associated to certain behaviors and to the species' visual ecology.

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