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Research Article

Eye Morphometrics and Retinal Organization in the Arboreal African Pangolin Supports Nocturnality

Adekanmbi A.J[1], Akin-Adigun O[2] and Akinola O.B[3]

[1]*Department of Anatomy, College of Medicine, University of Ibadan, Ibadan, Nigeria*
[2]*Department of Anatomy, College of Health Sciences, Bowen University, Iwo, Osun State, Nigeria*
[3]*Department of Anatomy, College of Health Sciences, University of Ilorin, PMB 1515, Ilorin, Nigeria*

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Abstract
The arboreal African pangolin Manis tricuspis is one of the eight extant specie of pangolins and is known for its presumably poor vision. Detailed measurements of its eyes and retinal cell organization are not available. This study describes the shape of its eyes and organization of its retina cells thereby bringing to light the actual implication of its observed visual adaptations. We conclude that the pangolin has evolved a visual mechanism that is attuned to a nocturnal lifestyle rather than being described as visually poor.

Key Words: *Pangolin, Eye, Adaptation, Retina, Organization*

INTRODUCTION

The visual system has been reported by several authors to evolve morphological variations in response to when they move around for survival (Hall et al., 2009). Increasing a species sensitivity to light by increasing eye size relative to skull size or decreasing reliance on vision as seen in most reported nocturnal species are some of the mechanisms that has been reported (Martin et al., 2007). Both of these aforementioned details involve morphologic changes in their eyes, orbit and brain (Iwaniuk et al., 2010). For example, the largeness of the cornea determines how much light enters the eye, and when they are really wide as previously reported in studied nocturnal species, they are better able to gather the sparse light available. On the other hand, the length from the maximum point on the cornea to the point of exit of the optic nerve determines the visual acuity of the eye. It should be noted that when this distance is long, the projected image on the retina gets larger hence increased visual acuity (Martin, 1993).

Nocturnal species need to increase their sensitivity for scotopic vision hence they evolve mechanisms that adopt corneas that are wide in comparison to their eye axial length as opposed to what is typically found in crepuscular and diurnal species (Corfeild et al.,2011). The aim of alterations in the size of the eye and its shape is to raise its sensitivity to the reduced amount of light that is available to them in their scotopic environment (Corfeild et al, 2011). It should be noted that this generalization give a good overview of visual morphological adaptation in nocturnal species especially with most studies conducted in birds. Whether this is true of all nocturnal species is still being researched across a wider scope of orders.

The arboreal African pangolins Manis tricuspis is one of the eight extant species of pangolins also known as the white bellied pangolin or three cusped pangolin native to West Africa and some parts of Central Africa (Schlitter, 2005). They are rare unique modern mammals that belong to the order Pholidota (Wang 2016). They are the only mammals with their head, trunk, tail, upper and lower extremities completely covered in hard keratinous overlapping scales, except for a small part of their ventral skin which is scales free (Kawashima et al., 2015). They have recently been displaced from their previous classification with Xenathra (armadillos, sloths and others) to the Clade Ferae class based on results from molecular phylogenic studies (Arnason et al., 2008; Meredith et al., 2011). Their vision was presumed to be poor (Mondadori 1988) until recent evidence from gross and microscopic studies changed the earlier presumptions. Their visual sense is now reportedly neither over nor under developed, based on a visual qualitative assessment of the visual system (Imam et al., 2017). The basic retinofugal pathways of Manis pentadactyla were described (Lee et al., 1991). They reported fibres projecting to the suprachiasmatic nucleus, lateral geniculate nuclei, pretectal area and superior colliculus were not considered to vary greatly from the retinofugal pathways of other mammals. The only feature that is unique so far reported is that their retinal cone photoreceptors fail to express the short wavelength cone opsin ad their superior colliculus seems to lack a laminar appearance (Adekanmbi et al., 2016). Despite these few reports about their visual system, there is still a lot to be understood about their visual adaptations. Here we report the morphometric analysis of their eyes to determine the degree to which they have adjusted their corneal diameter in relation to the axial length of their eyes as well as their retinal cell organization.

**Author for Correspondence:* +2348028940657 E-mail: *joandavid2011@gmail.com*

MATERIALS AND METHODS

The present study was approved by the Ethics Committee of the College of Medicine, University of Ilorin. A total of eight male adult arboreal African pangolins (Manis tricuspis) with average body weights of 1700 g and approximate length of 70 cm were used in this study. They were obtained from the wild in Asejire, Osun State, which is located in South-Western Nigeria. They were picked up from their tree homes in the wild in the morning. This is because they are usually sleeping and less likely to resist at this time. They would usually roll up in a ball like motion when they sense danger with the intention of outwitting their captors by this act. They were subsequently transported in standard sized cages to the laboratory. All animals were sedated with a lethal dose of chloroform and were perfused transcardially first with normal saline and then with paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4.

Eyes were removed shortly after perfusion, weighed, measured, pigment epithelium removed, post-fixed in 4 % paraformaldehyde and cryo-protected with 30 % sucrose for 2 -3 days. Morphometric Measurements were done according to the method used by Ritland (1982). Specifically the maximum corneal diameters, maximum axial lengths and maximum transverse lengths of the pangolin eyes were determined to the nearest 0.01 mm using digital callipers. Mean is presented as Mean ± Standard Error of Mean (SEM)

In this study, we defined corneal diameter of the eye as the distance across the eye from its medial border to its lateral border, eye axial length was defined as the distance across the eye from its corneal centre to its medial-most portion, just beside the exit of the optic nerve, while transverse diameter was defined as the width across the eye at the widest region of its equator (Martin et al., 2004; Corfeild et al., 2011; Iwaniuk, 2010; Lisney et al., 2012). Cryo-protection was followed by embedding in Tissue Freezing Medium (Triangle Biomedical). Serial sectioning of embedded retina tissue was carried out in a Leica Cryostat at 20 μm and picked up onto slides.

This was followed by successive incubation of serial sections in blocking solution (3% normal donkey serum and 0.3% Triton-X-100 in phosphate buffered saline) for 60 minutes at room temperature. The next stage was incubation in primary antibodies diluted in phosphate buffered saline which contained 3% normal donkey serum and 0.3% Triton-X-100 for 12–14 hours at 4oC. Tissue sections were washed in PBS twice. The same procedure was carried out in single labeling experiments, with section incubation in primary antibody immediately followed by secondary antibody. In control experiments, one or both primary antibodies were omitted and this resulted in immunolabeling by either one or no primary antibody. The antibodies used are Anti-Calbindin, Anti-Choline Acetyltransferase (Anti-ChAT), Anti-Cocaine amphetamine transcript (Anti-CART), Transcription factor Chx10 (Antibody Chx10), Insulin gene enhancer protein (Antibody -ISL-1), Antibody to Syntaxin-1, Tyrosine hydroxylase (anti-TH), Anti-Pax6.

Bipolar cells were detected with anti-Chx10 (Exalpha) in sheep in a working solution of 1: 300. Amacrine cells were detected with anti-Syntaxin-1 (Sigma, USA), in mouse with a working solution of 1:500, anti-TH (Millipore) in sheep with a working solution of 1:2500, anti-ChAT (Chemicon) in goat with a working solution of 1:200, anti-Pax6 (Sigma) in a mouse working solution of 1:500. Horizontal cells were detected with anti-Calbindin (Swant) in goat with working solution of 1: 4000. Retinal ganglion cells were detected with anti-CART (Phoenix Pharmaceuticals) in rabbit with working solution is 1: 2000. Alexa-conjugated antibodies (Molecular Probes) and Cy dyes were subsequently used to secondarily immunolabel tissue sections for 120 minutes at room temperature. Sections were then washed thoroughly in phosphate buffered saline. Cover slips were applied on tissue sections with biomedia mounting media and an Olympus FV1000 scanning confocal microscope (Olympus America Incorperated, NY, USA) was used for imaging the sections. Images of sections were acquired as a Z stack by collecting light from thin (1um apart) regions of interest with the 40 x objective, with contrast and brightness settings appropriate for filling the 8-bit coding image range recognizable by the FIJI software. The zoom of the acquired image was in a ratio of 1 : 1 and florescence images were acquired at 1024 × 1024 (pixels) saved as OIF files in order to make images recognizable by FIJI on image J (NIH, USA). All antibodies were aliquots previously stored at −20∘C before use (kindly provided by the Sanes lab, Harvard University, MA, USA). For the triple labeling experiments, we took into consideration the multiple antibody labeling approach by imaging on multiple channels set to capture the different emission wavelengths on the microscope.

RESULTS

The mean eye axial length, corneal length and transverse length of the sixteen samples are presented in Table 1. The Maximum axial length ranged between 0.8 and 1.0mm while the Maximum corneal diameter ranged between 2.3 and 3.7mm. The lowest and highest values recorded for the transverse length were 1.0 and 2.9mm respectively.

The Vertical sections of the retina in M. tricuspis immunolabelled with antibody to Chx 10, CART & Pax6 are shown in Plate 1 while Plate 2 shows the immunostaining pattern for calbindin which is expressed by horizontal cells in the inner nuclear layer (INL) and their dendrites in the inner plexiform layer (INL) communicating with amacrine cell dendrites within the same layer. In multiple labeling with calbindin, ChAT and Syntaxin – I; a pan amacrine marker, co-labeling of calbindin and ChAT was observed in the starburst amacrines in the INL while some were displaced to the ganglion cell layer (GCL).

Gross observations of pangolin eyes shown in Plates 3 and 4 reveal eyelid opening laterally positioned in their heads with their cornea overlying in a spherical fashion. The cornea formed a curvature over a spherical lens which appears to fill the majority of their eyes and the remaining space was occupied by the viscous vitreous humor. They had an open thick eyelid through which the eyes were seen in the palberal cavity

Table 1:
Measured morphometric parameters of pangolin eyes

Eye morphometrics	Sample size	Mean ± SEM
Maximum axial length	16	2.863 ± 0.1499
Maximum corneal diameter	16	1.175 ± 0.1176
Maximum transverse length	16	1.913 ± 0.2030

Mean is presented as Mean ± SEM

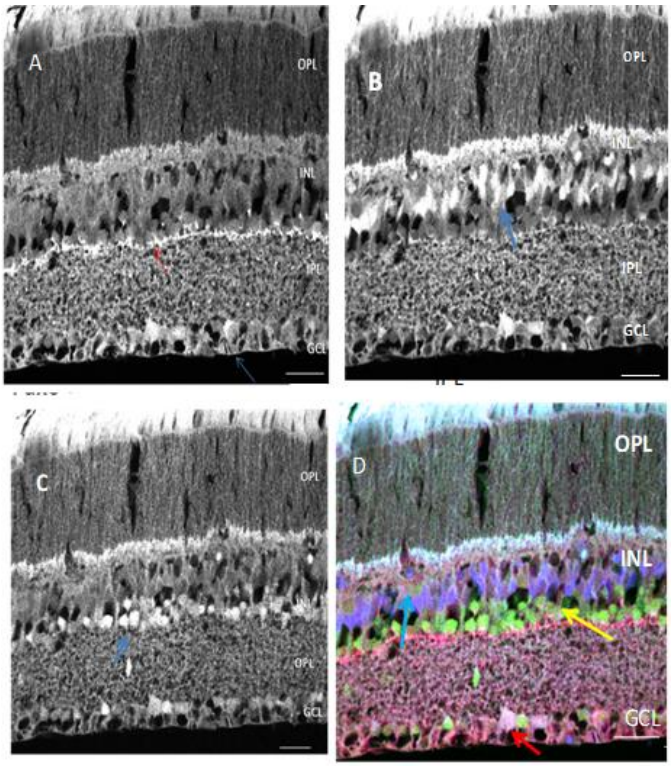

Plate 1
Vertical sections of the retina in M.tricuspis immunolabelled with antibody to Chx 10, CART & Pax6 at 20 □m magnification. A. CART demonstrating direction selective ganglion cells in the ganglion cell layer and their terminals in the outer part of the inner nuclear layer of the retina. B. Chx10 demonstrating bipolar cells in the inner nuclear layer. C. Pax6 demonstrating amacrines in the inner nuclear layer. D. Triple labelling with Chx10, CART and Pax6 to demonstrate the presence of bipolar cells, amacrines and retinal ganglion cell. OPL is outer plexiform layer, ONL is outer nuclear layer, IPL is inner plexiform layer, INL is inner nuclear layer and GCL is ganglion cell layer.

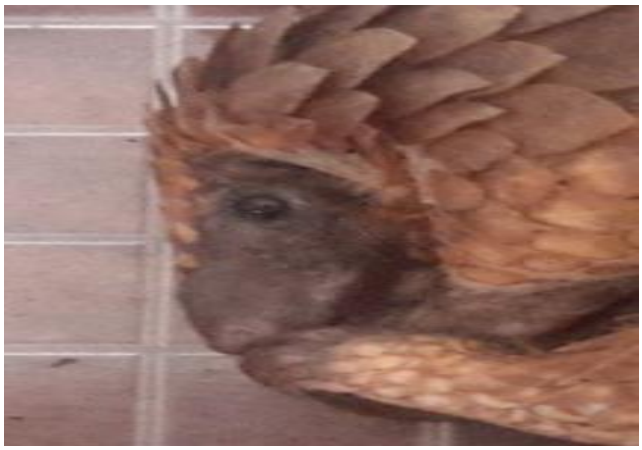

Plate 3
An Image of the pangolin eyes insitu

DISCUSSION

African pangolins are generally presumed to have diminished their reliance on vision and depend more on their sense of smell to find food (Mondadori, 1988). Results presented in table 1 as well as gross observations in Fig 4 indicate that they have adopted a visual system attuned to increased sensitivity to light with a cornea length that more than doubles their axial length, suggesting that their eyes are fashioned to gather light in dim environments. Generally, nocturnal animals have broader corneas, in relation to the axial length of their eyes when compared to either diurnal or crepuscular animals (Hall, 2009). The essence of this changes in eye size and shape is to facilitate sensitivity of the eye to the scarce quantum of light, often available to these animal species in their scotopic environment (Corfeild et al., 2011).

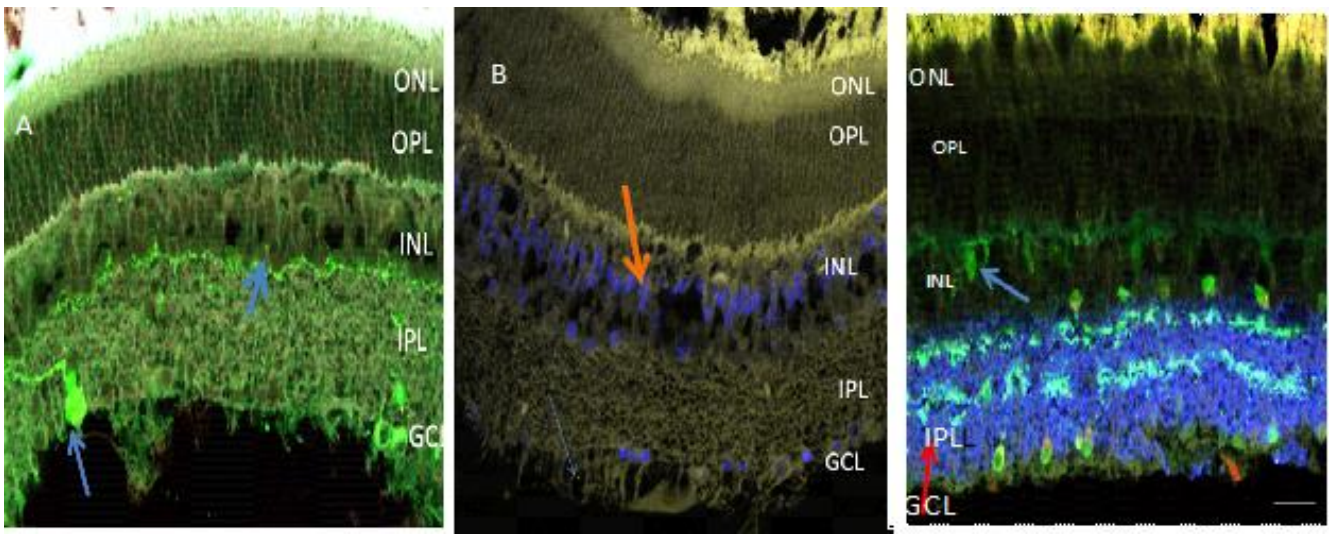

Plate 2
Vertical sections of the retina in M.tricuspis immunolabelled with single antibody to tyrosine hydroxylase (TH), islet and triple labelling for ChAT, Calbindin, Syntaxin at 20 □m magnification. A.TH demonstrating dopaminergic amacrines in the inner nuclear layer and retina ganglion cell layer and their terminals in the inner plexiform layer of the retina. B. Islet demonstrating amacrines in the inner nuclear layer and ganglion cell layer. C. ChAT demonstrating starburst amacrines and their terminals forming two bands in cyan colour in the inner nuclear layer and inner plexiform layer. Calbindin expression is shown horizontal cells and Syntaxin in amacrine cells and in their terminals. OPL is outer plexiform layer, ONL is outer nuclear layer, IPL is inner plexiform layer, INL is inner nuclear layer and GCL is ganglion cell layer

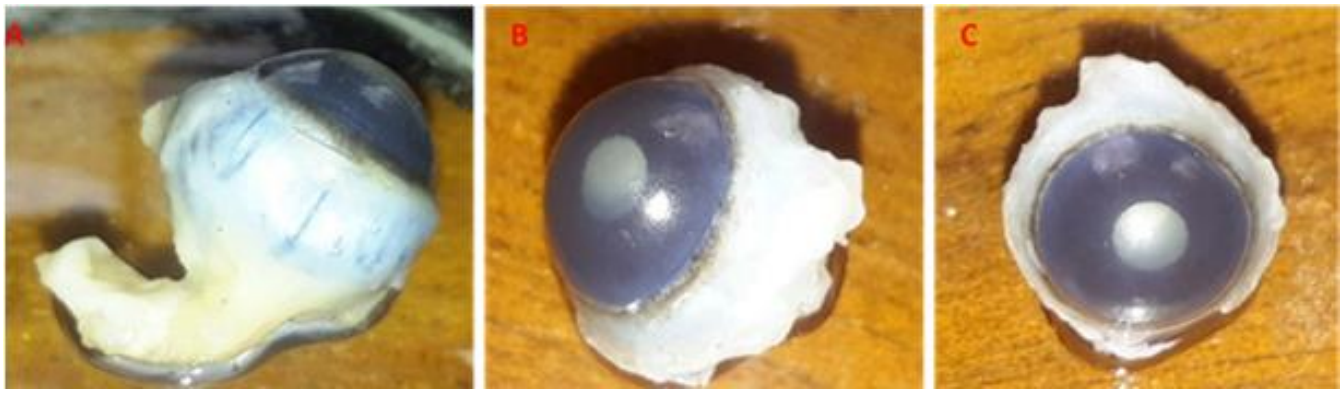

Plate 4
Photograph of excised pangolin eyes; A. Posterior-lateral view. B. Anterior-lateral view. C. Anterior view.

The essence of this changes in eye size and shape is to facilitate sensitivity of the eye to the scarce quantum of light, often available to these animal species in their scotopic environment (Corfeild et al., 2011). Studies among species belonging to different orders have reported that axial lengths of the eye is associated with visual acuity and specifically the fact that the longer the axial length, the bigger the image projected on the retina appears (Hall and Ross 2007; Hall, 2008). On the other hand, with a wide corneal diameter, the specie is likely to be nocturnal and their eyes designed to try to catch the few light photons available in the dark (Martin, 1993).

Presumably if all pangolins have poor eye sight, from an anatomical perspective, then their light gathering device; the cornea should be small in comparison to other ocular parameters. By this, we mean an eye shape with a small cornea diameter, relative to the axial length of the eye. We can also say it should evolve little change in cornea diameter in relation to its axial length, all of which should not really function much to facilitate vision. Our observations revealed an eye shape attuned to increased sensitivity in the arboreal African pangolin. The reduced axial length in comparison to corneal diameter observed implies that visual acuity is not a priority to them rather they are more interested in widening their eyes to catch the light available in the dark.

The current study also sought to characterize retinal neuron organization in the African pangolin using markers of terminally differentiated cells.

The circuitry or connection between horizontal cells that express calbindin and starburst amacrine cells that express choline acetyl transferase (ChAT) is intact in the retina of arboreal African pangolin. This statement is made based on our observation as shown in Fig 2 of the immunostaining pattern for calbindin which is expressed by horizontal cells in the inner nuclear layer (INL) and their dendrites in the inner plexiform layer (INL) communicating with amacrine cell dendrites within the same layer. In multiple labeling with calbindin, ChAT and Syntaxin – I; a pan amacrine marker, co-labeling of calbindin and ChAT was observed in the starburst amacrines in the INL while some were displaced to the ganglion cell layer (GCL). This kind of displacement has been reported in mammals that depend on their visual system rather than on their sense of smell or hearing (Eckenstein and Thoenen 1982). It should be noted that retinal calbindin has been studied across different orders, some of which include squirrels, rats, mudpuppy, rabbits, monkeys, cats and some other primates (Pasteeels et al., 1990; Cuenca et al., 2002; Peichl and Gonzalez-Soriano 1994). So far, most of the mammalian retinas that show this kind of retinal calbindin distribution have turned out to be the rod dominated retinas, (Wassle et al., 1995a; Goebel and Pourcho, 1997; Massey and Mills, 1996, 1999). This implies that it is likely that the African pangolin has a rod dominated retina. This is an adaptation for a nocturnal lifestyle because rods are essential for night time vision.

In general, the role of horizontal cells is to modulate the passage of information from photoreceptors to bipolar cells in the outer plexiform layer (OPL) (Peichl and Gonzalez-Soriano 1993). Based on studies on non-rodent species, mammalian retinas generally contain two types of horizontal cells; an axonless type called A- type and a short axon type called B-type (Peichl et al., 1998). The axonless horizontal cell type has a large less branched dendritic tree with fewer and stouter primary dendrites that makes synaptic contacts exclusively with cones. On the other hand, the short axon type horizontal cell has a small dendritic tree formed by many tiny dendrites that connect with cones and an axon that ends in a complex axon terminal system that connects exclusively with rods (Linberg and Fisher, 1988).We did not determine the horizontal cell type with the highest population in this study.

This study also demonstrated the laminar organization of ganglion, amacrine and bipolar cells in established levels in the retinas of visually dependent mammals. This was revealed by immunolabelling with anti- Islet 1 antibody. Islet 1 labels GCLs, amacrines and bipolars in mice. It is a highly conserved transcriptional regulator belonging to the family of LIM homeodomain-containg protein that facilitates cell fate decisions in different systems (Pfaff et al., 1996). In adults, retinal expression of Islet 1 is limited to ON bipolars, most amacrines and sub populations of ganglion cells (Galli - Resta et al., 1997). This implies that ON bipolars, possibly a lot of amacrines with at least some ganglion cells are present in the retina of the pangolin. Its pattern of expression appears to be consistent with previous reports across other vertebrate species (Elshatory et al., 2007; Alvarez- Hernan et al., 2013). Immunolabelling revealed an orderly array of intense multi-laminar staining in the mid section of the INL and more sparsely in the exterior of the INL. This is slightly different from what obtains in the mice, where the Islet 1 intense labeling is usually intense in the external region of the INL and the scattered array of cells are usually observed in the mid to inner section of the INL (Elshatory et al., 2007). This might have functional implication for retinal information processing. Islet did not co-localize with any other antibody correlating previous studies in mice (Marquardt et al., 2001; Elshatory et al., 2007).

Retinal bipolar cells are interneurons that transmit visual signals from photoreceptors to ganglion cells. Chx10 is a transcriptional regulator and is expressed in terminally differentiated bipolar cells (Hatekeyama et al., 2001; Elshatory et al., 2007). In this study, intense labeling was observed in bodies of cells found in the center region of the INL correlating with previous studies in mice (Elshatory et al., 2007; Voinescu et al., 2009). Bipolar cells are broadly categorized into ON-center and OFF-center categories based on their ability to depolarize in the case of the ON-center type or hyperpolarize (OFF-center type) in response to light (Nelson and Kolb 1983). Other classifications of bipolar cells are based on whether they make synaptic contacts or receive imput from cones or rods (Brown and Masland, 1999). We did not determine the types of bipolar most prevalent in this study but if our earlier presumption that the pangolin is likely to be a rod dominated retina, then it likely that there would be more rod bipolar cells in this Chx10 expressing cells rather than cone bipolars

Amacrine cells (ACs) are retina inhibitory interneurons and there are at least 30 different types (Macneil and Masland 1998). One way of classifying ACs is by morphology and the most important part of this classification is based on the IPL sub-lam¬ina in which their processes arborize and on the degree to which their arbors are in a tangential plane. Our result revealed that most of the pangolin dendrites terminated in sublamina 3 and 4.

Amacrines (ACs) subtypes are classified into wide/medium-field ACs, which use GABA as their neurotransmitter and narrow-field AC which use glycine, as their neurotransmitters, which is then usually accompanied with neuropeptide ie cotransmitter (Kay et al., 2011). Wide- and medium-field ACs project to individual sub-lamina of the inner plexiform layer (IPL) and mediate lateral interactions that modulate the receptive fields of the RGCs (Kay et al., 2011). They make up approximately twenty morphologically distinct subtypes (Macneil and Masland, 1998). Many subtypes have been identified in the retina of a variety of mammalian species (Masland and Macneil 1998). In contrast, the majority of narrow-field ACs, project to several IPL sub-lamina, facilitating vertical interactions across parallel circuits. There are further subdivisions within these broad categories which function in determining responses to specific visual features of appropriate RGCs (Kay et al., 2011).

In triple labeling with ChAT, Calbindin and Syntaxin, the terminals of the ChAT positive cells made two ChAT bands in the IPL dividing the inner plexiform layer (IPL) into sublamina as reported in mice retinas (Samuels et al., 2011).

ON-OFF direction selective ganglion cells (DSGC) were identified with the molecular marker CART; Ccocaine- and amphetamine-regulated transcript. Antibodies to CART labels approximately 15% of all retinal ganglion cells (RGCs) as well as a small group of nonstarburst amacrines in the inner nuclear layer. In this study, it labeled RGCs in the ganglion cell layer with projections to the ON and OFF sublamina in the IPL and amacrines in the INL as previously reported in other species (Rogge et al., 2008).

In conclusion, our results indicate that the African pangolins have widened corneas in relation to their eye axial length; an adaptation common to nocturnal species and they have a reduced eye axial length implying reduced image size on their retina. This suggests decreased reliance on visual acuity. Also they have a laminar organization of horizontal cells, amacrines and ganglion cells needed to maintain the circuitry requirement for the transfer of visual information as previously reported in species that are vision dependent. This implies that the African pangolin retina is consistent with what is found in typical visually guided species howbeit modified to suit its own needs, hence we disagree with the presumption that all pangolins generally have poor vision. The African pangolin has evolved some mechanisms for increasing their sensitivity to light in their scotopic environment, through increased cornea size, however they do not appear to favor visual acuity. This detailed analysis of the shape of the pangolin eyes and its retina organization describes the extent to which its visual adaptation has been affected by its nocturnal lifestyle. Further quantitative and behavioral experiments might give better insight into other visual adaptations that they may have evolved in response to their environment and time of activity.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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