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COMPARATIVE HISTOMORPHOMETRICAL STUDY OF RETINA IN INSECTIVOROUS BAT (*RHINOPOMA HARDWICKII* AND FRUGIVOROUS BAT (*ROSSETUS AEGYPTIACUS*))

Farangis Ghassemi and *Soghra Gholami

Department of Anatomical Sciences, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

*Author for Correspondence

ABSTRACT

The identification of organism is necessary in order to protect them, especially animals that have important role in the ecosystem. In this study, 5 male insectivore bats (*Rhinopoma hardwickii*) weighing of 8.1 ± 0.3 g and 5 male frugivorous bats (123 ± 0.8 g) captured by mist net from Tadvan cave (southern Iran). They were anesthetized and dissected their both eyes. The lens and vitreous humor of them were removed and remaining tissue were immersed in mixture of glutaraldehyde 4% and paraformaldehyde 2% (1 hour), rinsed with 0.1 molar sodium cacodylate buffer (for 2×20 minutes), and post fixed in osmium tetroxid 1%. The specimens were dehydrated, cleared, infiltrated with 1:1 propylene oxide and TAAB resin and embedded in pure resin. Semisections ($0.5\mu\text{m}$) were prepared (ultramicrotom), mounted, stained with 1% methylene blue and thickness of retinal layers were determined by eye piece micrometer. Obtained data were analyzed by SAS and Tukey's test ($p < 0.05$). The flat-mounted retina of *R. hardwickii* ($210.18 \pm 20.40\mu\text{m}$) was thicker significantly than the undulating form in *R. aegyptiacus* ($109.61 \pm 15.26\mu\text{m}$). In both of species, retina was duplex, rod – dominated and divided into 10 defined layers with various thicknesses. Also several types cell were observed in their inner nuclear layer and ganglion cell with different density and morphology were seen in every species. The results indicated that retinal structure in examined species was implemented with basic mammalian pattern, although the arrangement of its layers in *R. aegyptiacus* is unique. A few differences between them are due to adaptations to their lifestyle.

Keywords: Retina, Thickness, Nocturnal, Photoreceptor, Bat, Habit, Habitat

INTRODUCTION

Behavioral studies have shown that vision is important in various bat activities and they indicate differences depending on the species in visual capabilities (Orbach & Fenton, 2010; Boonman *et al.*, 2013). Retina, the sensitive tissue for visual recognition, is a thin layer that lines inside of eyeball. It receives light, converts it into neural signals, sends them to the brain via the optic nerve, and forms the image (Ebrej & Koutalos, 2001; Hoon *et al.*, 2014).

Retina in mammals is similar to other vertebrata, and their differences due to the adaptation with their habits and habitats such as cursorial, flying, aquatic, or burrowing environments (Eklöf *et al.*, 2014). Diversity of vision type in mammals causes difference in layer thickness and density and distribution of the retinal cell especially photoreceptor cells (Peichl, 2005; Saberi and Gholami, 2012).

Bats with more than 1300 known species are one of the largest orders in mammals which are able to fly actually (Petrov *et al.*, 2014). They divided into two suborders include microchiroptera and megachiroptera. *Rhinopoma hardwickii* is an insectivorous microchiropteran species (Figure 1a). It is a nocturnal bat with small eyes that adapted to life in dark site as caves. Due to poor eyesight, they use echolocation for feeding and perceiving its surroundings (Altringham and Fenton, 2004), nevertheless vision plays an important role during their activities (Eklöf and Jones, 2003).

Rousettus aegyptiacus belongs to megachiropteran bat and is known as the only frugivorous bat which is capable of primitive echolocation (Pettigrew *et al.*, 2008). So they make use of visual cues, smell and olfactory for plant detection spatially color fruits.

Retina in bat as other vertebrate has many different cells include bipolar cells, horizontal, amacrine, and ganglion cells, followed the general plan of mammals, but density and distribution of them are vary in

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different groups (Muler *et al.*, 2007). Some microbats such as *Rhinopoma* spp related to echolocation for object detection and orientation, some of them such as *Anthrozous pallidus* can detect objects by using vision alone and the others were intermediate between these two groups (Eklöf *et al.*, 2014). Due to the ability of migration and flight in bats, it is expected that vision is important for them. Color and even UV detection was reported for them (Jacobs and Rowe, 2004; Müller *et al.*, 2009; Xuan *et al.*, 2012).

The bat retina is similar to other mammals consisting 10 layers, but there are differences in structure of it that probably are related to adaptation with their habitats.

Many investigations related to vision have carried out about various species of vertebrata (Jacobs *et al.*, 2004; Hoshi *et al.*, 2011; Stephen *et al.*, 2014), microbat (Jacobs and Rowe, 2004; Müller and Peichl 2005; Eklöf *et al.*, 2014; Wang *et al.*, 2015) and megabat (Peichl, 2005; Müller *et al.*, 2007).

Although some information is available about retina in bats, due to high diversity in this order, more investigation in various groups is necessary. The other hand, more information about beneficial animals like bat helps to protect them, and also comparative study, present study, is a fruitful approach to explore the range of variation within the chiropteran retina and to identify species with different patterns is very useful.

MATERIALS AND METHODS

5 male microbats (*Rhinopoma hardwickii*) weighing of 8.1 ± 0.3 g and 5 male frugivorous bat (123 ± 0.8 g) were captured by mist net from Tadvan cave (southern Iran). They were transferred to the laboratory, anesthetized using ether, and dissected their both eyes. All the experimental procedures, in compliance with regarding the National Institute of Health for using the laboratory animals. Horizontal diameters of separated eye were measured and cut it in half at the optic nerve. The lens and vitreous humor of them were removed, and remaining tissue include sclera, choroid and retina immersed immediately in mixture of glutaraldehyde 4% and paraformaldehyde 2% for 1 hour. They were rinsed in 1% sodium cacodylate buffer (Ph= 7.3) for 2×20 min and post fixed in osmium tetroxide 1%. Then the specimens were dehydrated through a graded ethanol series (50, 75, 95,100%) 5 min for every grade. Then they were cleared with propylene oxide 100% and infiltrated with a mixture of propylene oxide and resin (TAAB) (1:1) (TAAB 812, DDSA, MNA, DMP30), incubated (65 C°) overnight, and embedded in pure resin. The semithin sections (0.5 μ m) were prepared by ultramicrotome (C. reichert, Austria om U3), mounted and stained with 1% methylene blue. The histomorphological study (Dino software) was carried out by binocular microscope in both two species. The total retinal thickness was measured from both sides of the optic nerve by using micrometer eyepiece in binocular microscope which was calibrated, and thickness of the retinal layers was compared in two species. Also their histological structure was examined by light microscope with 40 x to 100 x. Obtained data was analyzed with factorial analyses of variance in SAS and the thickness of layers were compared by using Tukey's test. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Results

The collected data in this study showed that although the diameter of eye in *R. hardwickii* (2.3 mm) was smaller than *R. aegyptiacus* (6.8mm) but total retinal thickness in *R. hardwickii* (210.18 μ m) thicker than the other species (108.61 μ m) significantly.

Retina in *R. hardwickii* (Figure 3a) was flat-mounted and in *R. egyptiacus* (Figure 3b) was undulating form, although inner layers, from inner plexiform layer to inner limiting membrane, approximately were linear (Figure 3b).

Also retina in both species was avascular, and includes two types of photoreceptor, rod and cone, with various densities (Figure 3). Their duplex retinas were divided to 10 defined layers with different thickness (Table I). According to analyzed data, every layer of retina in *R. hardwickii* was thicker than the same layer in *R. egyptiacus* significantly ($P < 0.05$), except pigmented epithelium layer (Figure 2). The thickness of retinal layers in two species was summarized in Table I.

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Figure 1: a- Insectivorous bat (*R. hardwickii*); b- Frugivorous bats (*R. aegyptiacus*)

Table 1: Comparative micrometric results of retina in *R. hardwickii* and *R. aegyptiacus* (Mean ± SD)

Thickness(μm) in <i>R. aegyptiacus</i>	Thickness(μm) in <i>R. hardwickii</i>	Layer	
9.29 ± 2.91	8.54± 0.64	Pigmented epithelium. L	1
16.56 ± 3.60	38.55 ± 4.05	Photoreceptor. L	2
2.95 ± 0.45	3.23 ±1.02	Outer limiting membran	3
14.75 ±0.50	40.38 ± 2.32	Outer nuclear. L	4
5.10± 1.94	4.94 ± 0.51	Outer plexiform. L	5
18.52 ± 1.55	41.79 ± 2.20	Inner nuclear. L	6
28.44± 1.55	41.70 ± 2.34	Inner plexiform. L	7
9.10 ± 2.30	16.41 ± 3.17	Ganglion cell. L	8
4.52 ± 0.43	13.54 ± 3.56	Nerve fiber. L	9
0.38 ± 0.03	1.10 ± 0.59	Inner limiting membrane	10
109.61±15.26	210.18 ±20.40	Total retinal thickness	

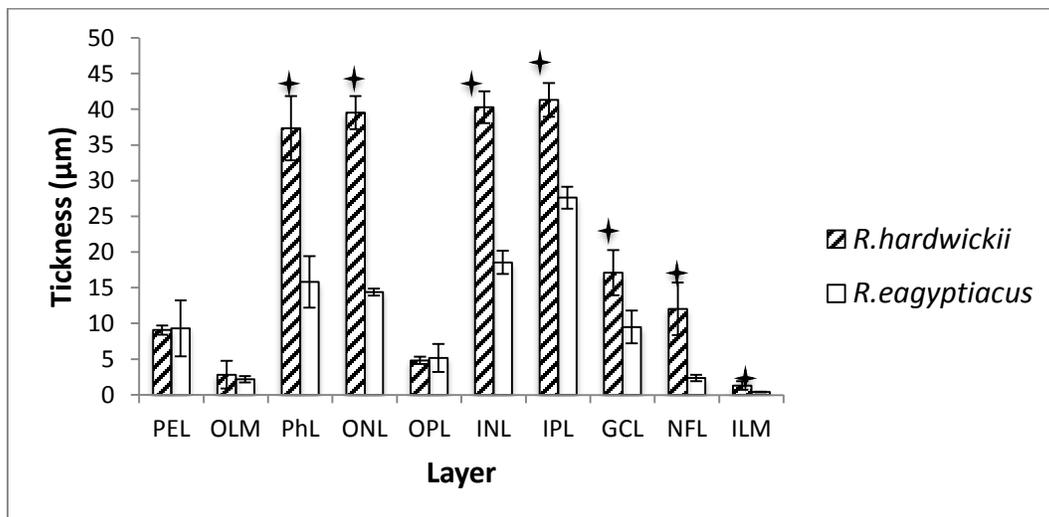
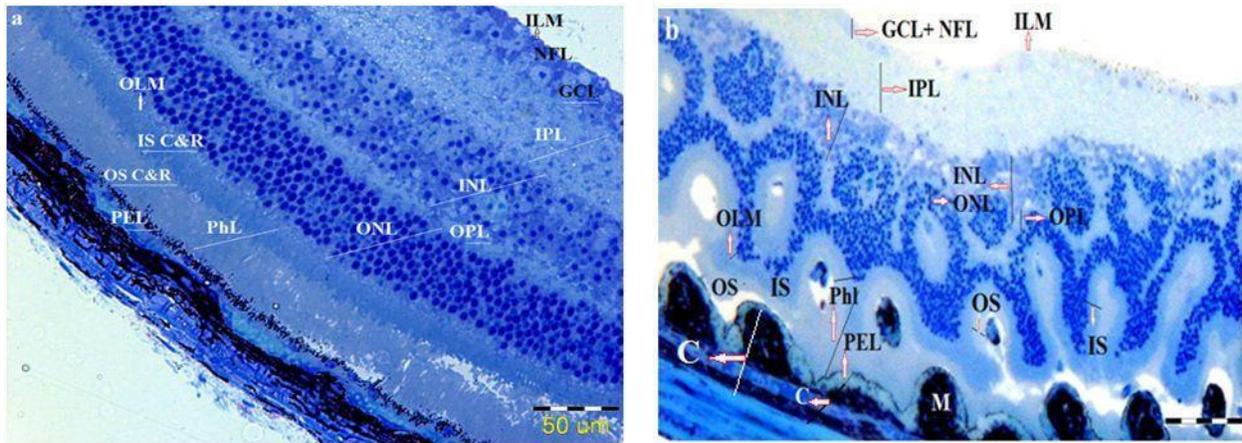


Figure 2: The thickness of retinal layers in *R. hardwickii* and *R. aegyptiacus* (Mean ± SD)

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Retina in both species was rod- dominant. Two types of photoreceptors (rod and cone cells),in mosaic pattern with different size of nuclei which located on different sites, were distinguished. The rods cells, which are very tightly packed, with elongated and dens nucleus. The cone cells with large and clear nucleus in low density were observed (Figure 3).

The pigmented epithelium layer (PEL) was thin, linear and consists of a single layer of large cells which attached at the choroid (Bruch’s membrane) and surrounded small melanosome densely in *R. hardwickii* (Figure 1a), while it was wavy shape with few pigmented cells (melanosome) in *R. egyptiacus*. Also some big pigmented granules of choroid extend toward photoreceptor layer (Figure 1b). Thickness of both nuclear layers (outer and inner) was similar nearly specially in *R. hardwickii* (Table 1). About eight to nine rows of nuclei were seen in outer nuclear layer which were arranged linearly in *R. hardwickii*, but four to five rows of nuclei with undulating arrangement or ring-shaped were in *R. egyptiacus* (Figure 2). Microanatomical details by light microscopy (40x -100x) are showed in Figure 3-5.



a: *R. hardwickii*

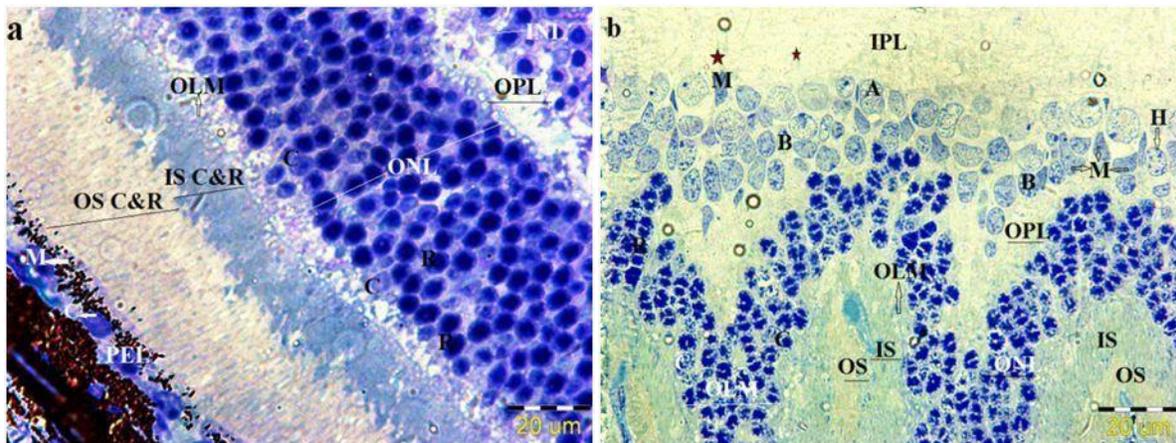
b: *R. aegyptiacus*

Figure 3: Retinal layers (staining: methylene blue, 40x & scale bar: 50 um)

Pigmented epithelium. L(PEL) Photoreceptor. L(PhL) Outer limiting membran (OLM)

Outer nuclear. L(ONL) Outer plexiform. L(OPL) Inner nuclear. L(INL) Choroidal (C)

Inner plexiform. L(IPL) Ganglionic cell L(GCL) Nerve fiber. L(NFL) Inner limiting membrane (ILM)



a: *R. hardwickii*

b: *R. aegyptiacus*

Figure 4: Retinal layers and cells (staining: methylene blue, 40x & scale bar: 50 um)

Inner segment of cone and rod (IS) Outer segment of cone and rod (OS) a: melanosom (M) b: Muller (M)

Outer nuclear. L(ONL) Outer plexiform. L(OPL) Inner nuclear. L(INL) Nerve fiber. L(NFL) Nerve (N)

Inner plexiform. L(IPL) Ganglionic cell L(GCL) Inner limiting membrane (ILM) Horizontal cell (H)

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Discussion

Vision is important for foraging and homing, and for predator avoidance. Although previous researches showed that megabats and microbats are monophyletic (Murphy *et al.*, 2001), but many differences in visual system were seen between them due to adaptation to various lifestyles that sometimes associated with different light conditions (Schwab and Pettigrew, 2005).

Differences in eye dimensions among the various species were reflected in retina size (Eklöf *et al.*, 2014), it is expected that vision in the megabat with larger eye which has a larger retina is better than microbat groups (Pettigrew and Manger, 2008). Although total thickness of retina in fruit bat, megabat, in this study was significantly ($p < 0.05$) less than microbat (Figure 2), due to undulating form of its retina (Figure 3-4), large area was occupied by retinal cells which are effective in its vision.

Probably thicker retina in *R. hardwickii* compensates its small eye. However, it seems that vision is more efficiency in undulating or ring form retina (*R. aegyptiacus*) compared with linear retina (*R. hardwickii*). Since a large number of photoreceptor cells can situate in the undulating layer, so that's a large number of short inner and outer segments of photoreceptors which are necessary for impulse transduction compensate their short segments (Lluch *et al.*, 2003).

The retinal thickness of megabat was reported up to 250 μm (Schwab and Pettigrew 2005), but the obtained result in our research ($109.61 \pm 15.26 \mu\text{m}$) demonstrated that vision in closely related to species adaptations to their niches.

Schwab and Pettigrew (2005) showed that in fruit eater bat such as *R. aegyptiacus*, choroidal papillae create a texture or undulations in the retina. This species is introduced as nocturnal bat however, it is able to fly and feed in highlight (Benda *et al.*, 2012). Hence expected to be significant population of cone cells in this species (Müller *et al.*, 2009), but rod cells were more than cones. It can be suggests that vision in this species is excellent compared with other species of bat (Pettigrew and Manger, 2008), meanwhile it is uniquely megabat that ables to echolocation (Holland *et al.*, 2004). This species like microbats prefers to stay in cave (dim light) to reduce predation risk in bright condition especially in dry and warm area (southern Iran), so as expected retina was rod- dominant in two species (Figure 2, 3). In addition the low density of ganglion cell (Figure 5) spatially in *R. aegyptiacus* confirmed this finding because rods synapse onto amacrine cells which contact both cone bipolars and ganglionic cells, but cones go to bipolar cell to ganglio cells directly. Thus the numbers of cone and ganglion cells are symmetric (Pittigrew *et al.*, 1988). The known cells that were seen in the ganglion cell layer (star in Figure 5a) may be ganglion cell (type 2) or neuronal elements that are similar to ganglion cell.

It has been reported that most bats have no cones, but the existence of it in examined species, *R. hardwickii* & *R. aegyptiacus*, in accordance with previous finding (Wang *et al.*, 2004; Müller *et al.*, 2007; Eklöf *et al.*, 2014) showed that they also have the ability of vision at photopic light levels.

All layers and cells that were observed in present research, were in general accordance with other mammals (Jacobs, 2004; Jacobs and Rowe, 2004; Stephen *et al.*, 2014), but there are the difference in arrangement, thickness and number of them (Eklöf *et al.*, 2014). Various animals have different image processing capabilities that were affected by their behavior, habits and ambience (Orbach and Fenton, 2010), therefore it can be expected to be difference in their retinal structure. In present study, the selected species live in common habitat but their habits are different, it seems that few difference which were seen related to their lifestyle and behavior.

The different types of photoreceptors, rod and cone, and long outer segment of the photoreceptors especially in *R. hardwickii* indicate that these species have color vision, and due to having large receptor area and mainly rod-based retina, their eyes well adapted to dim light (Bloomfield and Dacheux, 2001; Peichl, 2007; Muller *et al.*, 2007). The area of photoreceptor cell layer affects on vision since it contains inner and outer segments of photoreceptors which are necessary for vision. More length of outer segment includes more disks being full of opsin for absorbing photons to signal transduction and inner segments provide energy via their more mitochondria. The outer segments of cone are exposed to light during the day and rod outer segments act during the night, but high density of rod (Figure 4) which indicate a nocturnal adaptation is dominant.

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In contrast to the previous studies that have claimed that megabats have pure-rod retina (Rochon-Duvigneaud, 1943), duplex retina with rod-dominated was seen in *R. aegyptiacus* (Figure 3, 4) which is similar to *Rousettus madagascariensis* (Müller *et al.*, 2007). Probably it occasionally uses vision for prey detection under light condition by cone cell, and due to living in dimlight, the rod cells are considerably higher than cone cells (Figure 4). Also in *R. hardwickii*, vision and echolocation are used for object detection depending on its ability to contrast between the object and background (Boonman *et al.*, 2013). Previous studies have documented that visual acuity in bats varies and relates to light intensity (Eklöf *et al.*, 2014).

Although rod cell is sensitive in low light levels, some of diurnal mammals have rod-dominated retina (Peichl *et al.*, 2000; Jacobs *et al.*, 2003) and mammals with scotopic vision are mediated by rod photoreceptors dominant (Peichl, 2005).

According to micrographs (Figure 3), GCL and NFL in *R. hardwickii* are thicker and include many ganglion cells that probably match with thickness of their cell layers. Retinal ganglion by synapse with cone cells plays an important role in resolution (Heffner *et al.*, 2001). So high density of these cells and bundles of fibers in *R. hardwickii* are related to their nocturnal activity and insect detection on nights (Ebrey and Koutalos, 2001; Eklöf and Jones, 2003).

Conclusion

According to the results thus obtained, although the arrangement of retinal layers in *R. aegyptiacus*, megabat, is unique, yet its structure in both of the examined species followed basic mammalian pattern. Their eyes are functional not only in scotopic, but also in photopic vision. Despite having different diet and habit, the two species, due to common habitat (dark site as cave) have similar adaptations and are approximately alike. The few differences were restricted to layer thickness and density or distribution of cells. Therefore, for bat conservation, it is recommended that the caves and other their habitats of the bats must be protected.

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