

Morphological and Histological Investigation of the Ocular Structures in the Adult Insectivorous Bat (*Pipistrellus kuhlii*) in Nineveh, Iraq

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Abstract: Studying the anatomy of the eye in different organisms offers considerable insights into their evolutionary strategies and adaptations. The present work focuses on the examination of the ocular structure in *Pipistrellus kuhlii*, a common species of bat found in Nineveh, Iraq. Our research aimed to contribute to enhancing the understanding of how this bat has altered its visual system in response to its environmental niche. Twelve adult bats weighing 5.5 - 6g were captured alive. Bats were anesthetized, and eyeballs were enucleated from the orbit, maintained in 10 % formaldehyde, and then the histological analysis process was performed. The present study first indicated that the *Pipistrellus kuhlii* eye has quite elaborate adaptations, certainly connected with efficient foraging in low light conditions. The retinal structure is specific, with no folding, but features a high density of rod cells for dim light and tracking prey during nocturnal activities. Second, a quite different layout of photoreceptor cells (cones) concerning color vision. These findings would challenge the general idea that bats have very limited color vision. The current research may offer useful information on the complex visual adjustments of flying mammals and increase our understanding of bats' vision.

Keywords: Bat vision, eye structure, flying mammals, foraging adaptations, *Pipistrellus kuhlii*.

Irak, Musul'daki Yetişkin Insectivor Yarasanın (*Pipistrellus kuhlii*) Göz Yapılarının Morfolojik ve Histolojik İncelemesi

Öz: Farklı organizmaların göz anatomisinin incelenmesi, evrimsel stratejileri ve adaptasyonları hakkında önemli bilgiler sunmaktadır. Bu çalışma, Irak'ın Musul bölgesinde yaygın olarak bulunan bir yarasa türü olan *Pipistrellus kuhlii*'nin göz yapısının incelenmesine odaklanmaktadır. Araştırmamız, bu yarasanın görsel sistemini çevresel nişine nasıl uyarladığını anlamaya katkı sağlamayı amaçlamaktadır. 5.5 - 6 gram ağırlığında 12 yetişkin yarasa canlı olarak yakalanmıştır. Yarasalara anestezi uygulanmış ve gözler yuvadan çıkarılarak %10 formaldehit içinde muhafaza edilmiştir, ardından histolojik analiz süreci gerçekleştirilmiştir. Bu çalışma, ilk olarak *Pipistrellus kuhlii*'nin gözünün, düşük ışık koşullarında etkili avlanmaya kesinlikle bağlı olan oldukça gelişmiş adaptasyonlara sahip olduğunu göstermiştir. Retina yapısı, katlanma olmadan, düşük ışık için yüksek yoğunlukta çubuk hücrelerine sahip olup, gece aktiviteleri sırasında av takibi için de özelleşmiştir. İkinci olarak, renkli görme ile ilgili fotoreseptör hücrelerinin (koniler) oldukça farklı bir düzeni ortaya konmuştur. Bu bulgular, yarasanın renkli görmelerinin çok sınırlı olduğu yönündeki genel fikirleri sorgulayacaktır. Şüphesiz, mevcut araştırma, uçan memelilerin karmaşık görsel ayarlamaları hakkında faydalı bilgiler sunabilir ve yarasa görmesi hakkındaki anlayışımızı artırabilir.

Anahtar kelimeler: *Pipistrellus kuhlii*, yarasa görmesi, göz yapısı, uçan memeliler, avlanma adaptasyonları.

1. Introduction

The anatomy of the eye in vertebrates, along with a review of vision in different animals and various species, has been reported for the fruit and flying foxes bats (Graydon et al., 1987; Suthers, 1970), fishes (El-Bakary & Abumandour, 2017), and hagfish (Dong & Allison, 2021). The order Chiroptera bats are considered the second largest mammalian order, constituting more than 1400 globally known species (Burgin et al., 2018; Wilson et al., 2015). Evolutionarily, Chiroptera is divided into two suborders: Megachiroptera has large-sized bats with big eyes (Boonman et al., 2014); the second is Microchiroptera with small-sized bats, small eyes but with poor eyesight (Greiter & Firzlaff, 2017). These bats show special behavioral adaptations with roosting habits in foliage, tree hollows, caves, rock crevices, and a variety of artificial structures (Jones et al., 2009). In fact, bats are active through the night when they go to feed on diverse kinds

of food such as seeds and nectar, fruits, rodents, blood, insects, fish, and frogs (Kunz et al., 2011). In this sense, bats are presented as the only mammals that can actively fly and play a vital role in the dispersal of plant seeds and pollination (Zhao, 2020). Owing to these unique adaptations to a wide diversity of habits and habitats, bats possess new insights following the growth of the echolocation system process (Teeling, 2009). Therefore, understanding the systematic development and biological activity of bats is critical as these wildlife play a vital role in maintaining the balance of both the ecosystem and health. Adult bats typically have dimensions that are evolutionarily matched to their weights, sizes, and shapes (Fenton, 2003). They show great ecological diversity and a considerable physiological pattern (Soliman & Emam, 2022). Most bat species use echolocation for hunting, navigation, and orientation to locate prey while foraging in the dark (Gutierrez et al., 2018; Warnecke et al., 2018) but they also utilize their eyes for particular visual

recognition (Rodríguez-Herrera et al., 2019). Moreover, frugivorous and nectarivorous bats can recognize the brightness and categorize the spectral alignment of light in the natural sources (Müller et al., 2009), which makes bats' vision dichromatic and UV-sensitive cones (Gorresen et al., 2015; Müller et al., 2009). The gross morphology and histological examination studies on those eyes proved that there are several specific differences in the retina regarding the density, retinal cell distribution, and layer thickness (Müller et al., 2013). The structure of eyes from several species and genera of megachiropteran and microchiropteran bats was described in some studies such as Pteropus species (Pedler & Tilley, 1969; Suthers, 1970), Frugivorous Bat (*Rousettus aegyptiacus*) (Aboelnour et al., 2020; Gholami & Ghasemi, 2021), and Egyptian insectivorous bat (Aboelnour et al., 2020). The histological structure of eyes in bat species demonstrates remarkable adaptations to their ecological niches, particularly in Phyllostomidae: Stenodermatinae (Antonio et al., 2021), fruit bat (*Eidolon helvum*) (Peter-Ajuzie et al., 2022), and the Egyptian fruit bat (*Rousettus aegyptiacus*) (Aboelnour et al., 2020). The cornea of these mega chiropterans is composed of thickness in the central region, a simple squamous tissue posteriorly, and a non-keratinized stratified squamous epithelium anteriorly, with a significant presence of fibrous stroma. The sclera is characterized by irregular connective tissue linked with the corneal stroma. A distinctive characteristic in these megabats is the choroidal papillae, which plays an optical role by reflecting the light onto adjacent areas of the retina, enhancing night vision and reducing dependency on echolocation. Furthermore, the retina is divided into outer layers such as the photoreceptor layers and retinal epithelium, and inner layers including ganglion cells and the inner nuclear layers. The histochemical analysis of the eye revealed the presence of mucins, glycogen, proteoglycans, glycoproteins, and hyaluronic acid in the cornea, retina, sclera, and choroid tissues, demonstrating particular adaptations for scotopic vision (Peter-Ajuzie et al., 2022). However, the retinal characterization of eyes for Iraqi insectivorous bats (*Pipistrellus kuhlii*, also known as *P. kuhlii*) is still not available in the literature, especially in Mosul city. In this respect, the present work is a unique study in Iraq to investigate the microscopic anatomy of various ocular tissues including the cornea, retina, and lens of *P. kuhlii*, reveal specific histological basis of low-light adaptations in the visual system of this bat species, and further the general knowledge of mammalian sensory development. It is worth mentioning that *P. kuhlii* belongs to the Vespertilionidae family that is commonly distributed in most of Europe (Benda & Ševčík, 2020) and in the Middle East.

2. Materials and Method

2.1. Selection of Animals

Bats were acquired from the local area in Mosul city. 12 male and female adult bats, with an average body weight ranging between 5.5 - 6g and a body length of 4.6 cm, were captured alive at dusk using mist nests in May and June 2023. The species selected for the present study was *P. kuhlii*, as this bat is the representative of insectivorous species found in Iraq. Only healthy bats were used in the current work to prevent any disease or confounding impacts of age on the histological analysis.

2.2. Anesthetization and Sample Collection

The selected bats were placed into a small glass with a tight-fitting lid. Using a syringe, 500µl of chloroform (N01AB020) was inserted into the chamber containing a gauze. Bats were gently placed inside the chamber for 1-2 minutes. All eyeballs were immediately enucleated from the orbit by lateral incisions using mini dissecting forceps, Adson 12 cm-4 ¾ length, under sterile circumstances to avoid any damage that may occur to the ocular structures. Eyes were instantly kept in a fixative solution for routine histological analysis. At the same time, other tissues, such as the fascial sheath, muscles of the eyeballs, and orbital fat, were taken out and discarded.

2.3. Fixation

The collected twenty-four eyes were fixed in 10% formalin solution for 12 hours at room temperature. After that, the eyes were transferred to the phosphate-buffered saline pH 7.4 and kept at 4°C for the next processing. Fixation is for the preservation of cellular structures and allows for penetration into the tissue to ensure proper staining reagents.

2.4. Tissue Processing & Embedding

After the fixation step, samples were carefully dehydrated through ascending sequences of alcohol solutions (Scharmin, Spain) ending in 100%, then cleared in xylene to remove any lipids and enhance the tissue's pellucidity. The processed tissues were carefully mounted in a pure paraffin wax (Labssco Mennerert 854 schwabch) at a 56°C melting point via an automatic processor. The blocks of embedded tissue were left at room temperature to cool and harden.

2.5. Sectioning

Sections of 5µm thickness were prepared by a rotary microtome (Leitz wetzerm, Germany), followed by floating on a water bath (Kavi-kolb, Mormmert, Germany) (approximately 45°C) to align and straighten the tissues prior to placing them onto microscope slides, CAT No. 7101. The slides with tissues were then dried at 37°C for about 24 hours to facilitate the fluid repellent and enhance the adhesion of the tissue.

2.6. Staining

These sections were stained with Hematoxylin Eosin (H&E), Lap, UK. Slides were stained in Hematoxylin for 10 minutes and then rinsed in running tap water for 2 minutes. Slides were differentiated in 1% acid alcohol for a minute, then rinsed in tap water for 2 minutes. Slides were dipped in ammonia water for 20 - 60 seconds and rinsed in tap water for 2 minutes. Next, slides were stained in Eosin Y for 2 minutes and then rinsed quickly in distilled water to remove excess eosin. Subsequently, the selected slides were dehydrated in ethanol solution at increasing percentages (70%, 90%, and 100%), then cleared in xylene and mounted with a coverslip by DPX.

2.7. Microscopy & Imaging

The sections were tested by a light microscope (Olympus BX51) with different magnifications (10x, 40x, and 100x) to obtain a detailed evaluation of the structure of the eye's components. A digital camera (Olympus Om-Japan) linked to the microscope was used to get high-resolution

images.

2.8. Histological Analysis

For the histological examination, the present study focused on different aspects as follows:

Cornea: the presence of the epithelium and the evaluation of collagen fibers within the stroma.

Lens: Examination of the lens capsule, lens fibers, and any possible anatomical alterations associated with other mammals.

Retina: Comprehensive analysis of retinal layers including internal and external nuclear layers, photoreceptor layers, and ganglion layers as well as the distribution of rod and cone cells.

Sclera: Evaluating the collagen arrangement and identifying particular features including the lamina blood vessels and cribrosa.

2.9. Ethical Considerations

All experimental procedures involving the handling and euthanasia of animals were carried out according to the

Instructions of the Institutional Animal Care and Use Committee (IACUC). The protocol number was UM.VET.2023.148, authorized on May 01, 2023, by the College of Veterinary Medicine, University of Mosul.

3. Results

The anatomical examination of the *P. kuhlii* eyeball showed that this species exhibits small spherical eyes, frontal, and coincides with its small body size (Fig. 1A & B). Macroscopically, it was confirmed that the *P. kuhlii* eye structure conforms to the general mammalian scheme. The eyes are divided into the fibrous tunic, vascular tunic, inner tunic (retina), and nerve fibers layer. The fibrous tunic includes the transparent cornea and opaque sclera, middle vascular tunic, or uvea, which is traditionally composed of three areas from the front to back: iris, ciliary body, and choroid. Most of the eyeballs were covered with the sclera, specialized for the transparent cornea, except for the front areas. It is composed of dense connective tissue and collagenous fibrous tissue, which give it durability and strength (Fig. 1C). An extraocular muscle is attached to the outer surface of the fibrous tunic. It thus probably helps the outer surface strongly control the movement of the bat's eye in different directions.

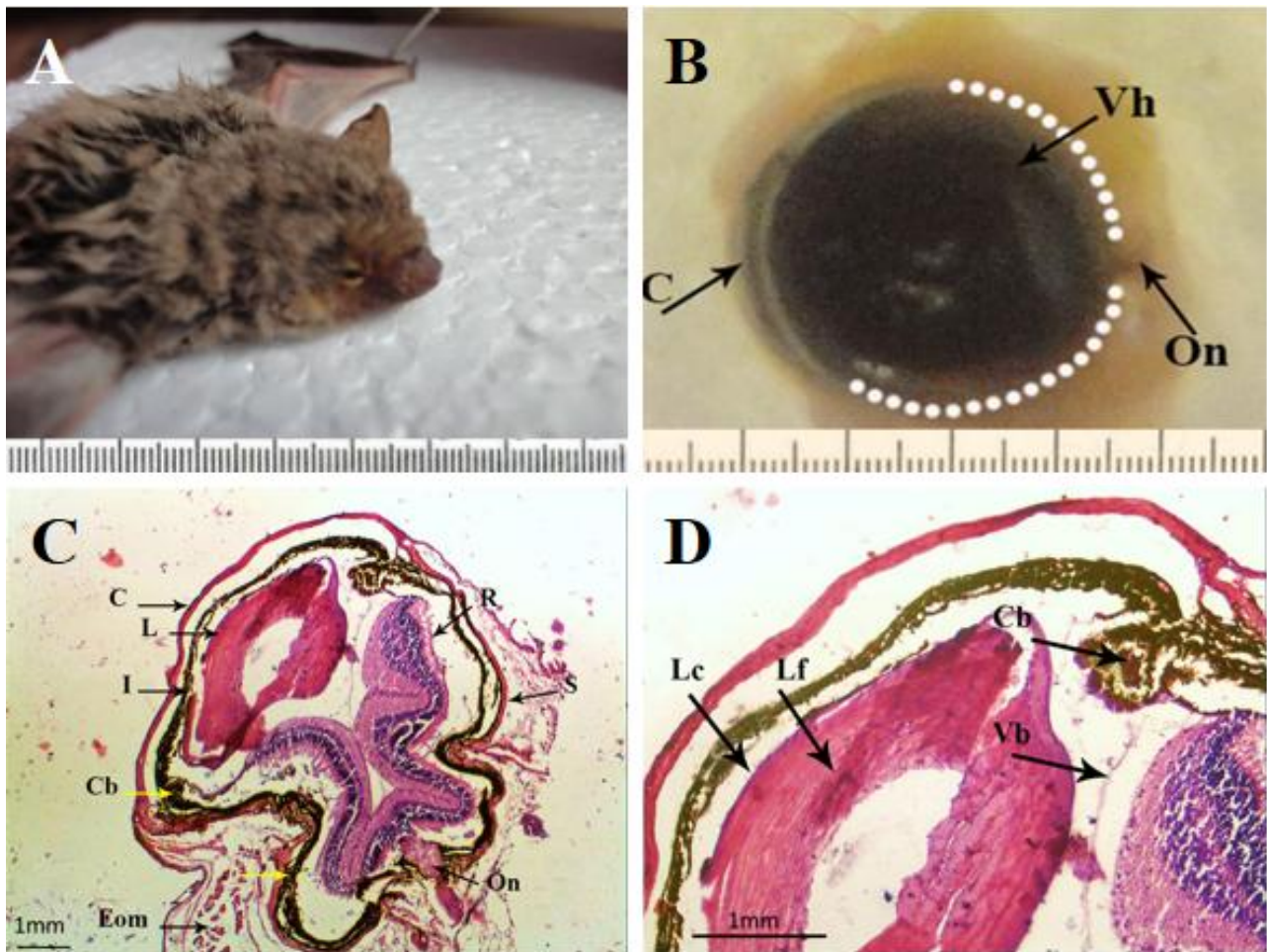


Figure 1. The morphological and histological structure of *P. kuhlii* eye. A shows the location of small spherical eyes. B, eye anatomy (obj in cm), C, Sagittal section (7 μ m) throughout the eye showing the structural features (40X). D, photomicrograph of bat eye at (100X). C: cornea, Vh: Vitreous humor, On: Optic nerve, L: Lens, I: Iris, Cb: Ciliary body, Ch: choroid, Eom: extraocular muscles, R: retina, S: sclera, Lc: Lenticular capsule, Lf: Lenticular fibers, Vb: Vitreous body. (C obj.40x, bar = 1mm; D: 100x, bar = 1mm. H&E stain).

Concerning the lens structure (Fig. 1D), it was convex, thinner at the edges and thicker at the center, found in front of the vitreous chamber. The lens was

composed of layers of specialized cells called lens fibers. These fibers showed arrangement in a compact, tightly packed manner, maintaining the lens's shape and

transparency. Additionally, the histological analysis found that the lenticular capsule, also known as the lens capsule, is an elastic, transparent, and fibrous membrane that surrounds the lens of the eye, primarily consisting of collagen fibers which are responsible for maintaining the shape of the eye and also the position of the lens within the eye.

As mentioned previously, the retinal tissue of *P. kuhlii* showed some similarities with that in the mammalian pattern. However, the findings of the current study showed other histological differences in the description of the retina and choroid. The differences may be directly related to the lifestyles of bats along with a variety of light conditions. The choroid was composed of a deep, smooth, pigmented layer. The retina of *P. kuhlii* also showed two macroscopically distinguishable parts: one region is present in the vast majority of the retina,

which is the pars optica (Fig. 2E), while the other was pars ceca, typical for the peripheral portions of the retina far from the central. Instead, it usually atrophies, or in some species, it may persist throughout life into a non-pigmented or spotted or mottled region completely devoid of cells. As shown in Figure 2F, the *P. kuhlii* species has a typical and regular structured retina; it has a higher thickness and invaginates the posterior chamber of the eye internally. Moreover, the present study showed various layers. The outer nuclear layer (ONL) consists of specialized photoreceptors (PS) that make rods and cones, the inner nuclear layer (INL), the inner plexiform layer (IPL) with Muller's cells, and ganglion cell layer (Fig. 2G). The ONL of the retina consists of a retinal pigmented epithelium which is a single layer of flat pigmented cells. ONL mainly contained the nuclei, cell bodies of rods, and probably cones. The domination of rod cells is interesting, and no specific folding was seen in the retina.

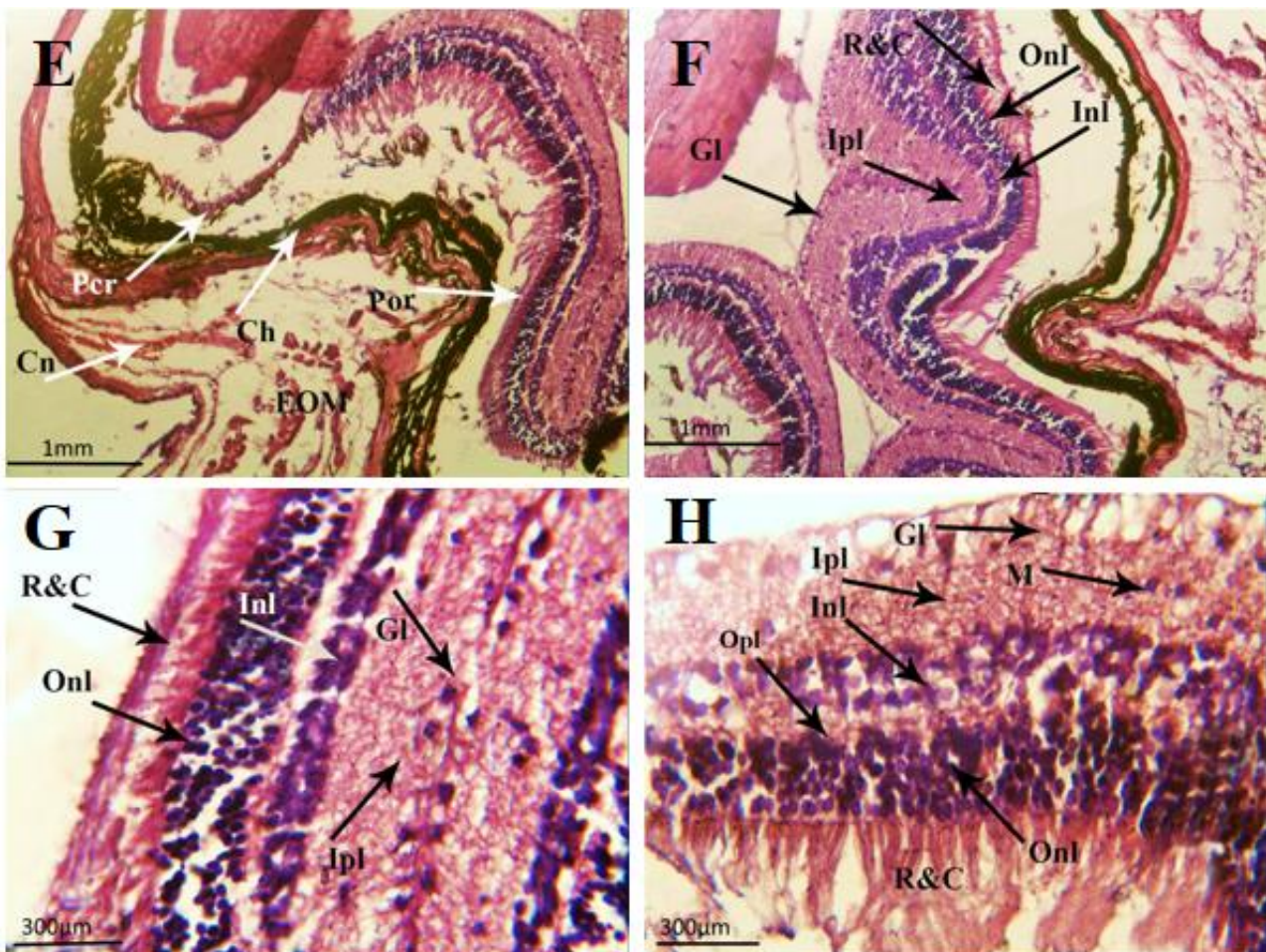


Figure 2. The histological structure of the eye in *P. kuhlii*. E, photomicrograph shows the Pars ceca retinae (Pcr), choroid (Ch), Pars optica retinae (Por), Extraocular muscles (EOM), Conjunctiva (Cn). F and G: photomicrographs (obj.100x, bar = 1mm) show the layers of retinae, represented by Rods and cones (R&c), Outer nuclear layer (Onl), Inner nuclear layer (Inl), Inner plexiform layer (Ipl), Ganglion cell layer (Gl). H: photomicrograph of bat eye focuses on Muller cells (M) and Ganglion cell layer (Gl) (G and H obj.400x, bar = 300µm, H&E stain).

Regarding the outer plexiform layer, it was thin and played only as a barrier between the INL and ONL. Besides, the IPL consists of axons and dendrites of ganglion cells and some of Muller's cells. (Fig. 2H).

4. Discussion

Pipistrellus kuhlii, also known as *Kuhl's pipistrelle* or *microbat*, is an interesting topic to discuss due to its small size and agile flight. *P. kuhlii* is currently the only

representative of the bat genus in Iraq. Thus, the eyes of this bat species are intriguing subjects to study and are probably the first attempt to describe the structure of the eye in this local bat. It is worth noting that while *P. Kuhlii's* eyes are well-adapted for nocturnal vision but they may not serve as effectively in bright daylight. Bats' pupils are relatively small and this characteristic does not constrict as in diurnal species, thus limiting bats' ability to regulate the amount of light that enters the eye. *P. kuhlii* possesses

unique ocular characteristics that contribute to its remarkable vision. Nocturnal adaptations of eyes seem well-adapted for nocturnal vision but may not be readily apparent in bright daylight. This adaptation suggests that bats prefer dimly lit environments as not to tire easily which in turn explains their predominantly nocturnal behavior. Although the eye structure of *P. kuhlii* was generally in line with that of other mammals (Baden & Osorio, 2019), some differences in the cells, thickness, and arrangement of layers were shown. The consensus from both small and large bats is that vision contributes significantly to predator avoidance, homing, and foraging. However, earlier findings did point to both small and large bats belonging to the same family (Murphy et al., 2001) with numerous variations apparent in the visual system between them due to their adaptations to lifestyles and varying light conditions (Schwab & Pettigrew, 2005). Notably, *P. kuhlii* eyes were relatively small and situated on the front of the head, considering body size. This adjustment hints at dependence on visual cues for migration and hunting under low light, a common nocturnal condition. It is one aspect that may justify using different visual communication channels.

A few studies have indicated that insectivore bats employ visual ability and echolocation for foraging and perching because of their eye size (Boonman et al., 2014). The low eye: brain mass ratio might be acting as a constraint in the mediation of visual acuity (Corral-López et al., 2017) indicating that bat eye size is directly related to the body size since plenty of others stated that the encephalization quotient, not the body size, is related to eye size (Burton, 2008; Howland et al., 2004). Obviously, behavior and lifestyle play an important role in determining the shape and size of the lens and the cornea of the organism (Burton, 2006; Thomas et al., 2006) for increasing light-gathering capacity, enhancing the bats' ability to track prey during twilight and night-time hours. It is worth noting that while *P. kuhlii*'s eyes are well-adapted for nocturnal vision but they may not perform as effectively in bright daylight. The present work indicated that *P. kuhlii* has a deep smooth pigmented layer and this is in line with Aboelnour et al. (2020) who showed that the choroid and retina are separated by a very thin layer of extracellular Bruch's membrane which serves a selectively permeable membrane between the choriocapillaris and retinal pigment epithelium (Hammadi et al., 2023). To the best of our knowledge, this study has demonstrated several features in the retina of an Iraqi insectivorous bat. The retina, located in the innermost layer of the eye, consists of two parts: a sensory portion and a non-sensory portion. The non-sensory one began at the ciliary body and extended to cover the ciliary body and iris. The sensory part of the retina was composed of specialized PS called rods and cones, ONL, INL, and IPL, with Muller's cells and the ganglion cell layer. Interestingly, the present work showed a strongly rod-dominant but poor pigmentation with a thin layer that is in accordance with the result of Aboelnour et al. (2020). The earlier study informed that the insectivorous bat *Rhinolophus capensis* has a normal retinal structure with no folding compared to the fruit-eating *Rousettus aegyptiacus* which achieves a unique feature in its retina to have a wide surface area and probably enhances scotopic vision (Bojarski & Bernard, 1988). Based on the current results, *P. kuhlii* exhibited a high density of rod

cells in their retinas. This domination of rod cells suggests that this bat has excellent scotopic vision to navigate through their environment or locate small insects in the dark since the rod cells detect light and are particularly sensitive to dim illumination. The distribution of these cells has been observed in different types of bats (Butz et al., 2015; Jeong et al., 2018; Müller et al., 2013). This arrangement is strongly linked to bats' behavior and surroundings (Jeong et al., 2018). Our results were in agreement with those findings documented by Aboelnour et al. (2020) who reported that rods' density in the retina of insectivorous bat species is higher than that in fruit bat species. Although the rods are the dominant PS in the retinae of both species, the cones' PS were found in both retinae. While rods are the major PS in the retinae of *P. kuhlii*, cone PS were also found in the retina of the same species. Besides its retinal structural role in *P. kuhlii*, the eyes of this bat revealed a specialized area of the retina, "Pars ceca retinae and Pars optica retinae," depending on their localization; probably, these areas are responsible for the high-resolution vision, capturing and processing visual information. The features observed in the eye of *P. kuhlii* bats highly optimize their vision under scotopic conditions and make them very effective hunters. Some researchers reported the presence of cone cells in megabats (Fujun et al., 2012; Peter-Ajuzie et al., 2022) as well as micro-bat species (Müller et al., 2009). A number of authors agree with the current work that rod cell densities in bats are a result of nocturnal or crepuscular activities. The present study also clearly showed extraocular muscles, apart from connective tissue, which resemble those of other mammals. The coordinated function of these muscles allows bats to perform eye movements in different ways such as lateral movement, gaze upward and downward, and rotation. These are necessary for visual orientation and balance in the air during flights especially while hunting and moving in the surroundings.

5. Conclusion

Pipistrellus kuhlii bats revealed remarkable adaptations for nocturnal vision in their eyes. They showed no folding in the retina with a high density of rod cells which all contribute to the bat's exceptional visual capabilities in low-light conditions. Further study about *P. kuhlii*'s eyes may open the door to the interesting world of nocturnal vision and adaptations that species developed to live normally in the dark.

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Ethics committee approval: All experiments involving handling and euthanasia of animal were approved according to the university committee of animal care and the use of animals in study.

Conflict of interest: The authors have declared that no conflict of interest.

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