

Original Article

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Interspecies comparative morphological evaluation of the corneal epithelial stem cell niche: a pilot observational study

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ABSTRACT

Background: The corneal and limbal morphology relevant to corneal epithelial maintenance in ten different species was examined using histological methods.

Objectives: The presence of a Bowman's layer, limbal epithelial cell, and superficial stromal morphology was examined in the following species to evaluate the differences in corneal thickness and epithelium: Java sparrows, frogs, macaws, spoonbills, red pandas, penguins, horses, Dobermans, orangutans, and humans.

Methods: Corneal sections (4 μ m) were obtained from ten ocular globes from three different animal classes: Aves, Amphibia, and Mammalia. All sections were stained with hematoxylin and eosin and periodic acid-Schiff reaction. After microscopy, all stained slides were photographed and analyzed.

Results: Significant morphological differences in the corneal and limbal epithelia and their underlying stroma between species were observed. The number of corneal epithelial cell layers and the overall corneal epithelial thickness varied significantly among the species. The presence of a Bowman's layer was only observed in primates (orangutans and humans). Presumed supranuclear melanin caps were noted in four species (orangutans, macaws, red pandas, and horses) in the limbal basal epithelial layer (putative site of corneal epithelial stem cells). The melanin granules covered the apex of the cell nucleus.

Conclusions: Supranuclear melanin capping has been described as a process within the epidermis to reduce the concentration of ultraviolet-induced DNA photoproducts. Similarly, there may be a relationship between limbal stem cell melanin capping as a protective mechanism against ultra-violet radiation.

Keywords: Melanin caps; supranuclear; UV light; cornea; eye

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Conflict of Interest

The authors declare no conflicts of interest.

INTRODUCTION

The cornea is the transparent front of the eye, and its clarity is vital for the transmission and refraction of light to the back of the eye for visual perception (**Fig. 1**) [1]. The human cornea is composed of five main layers: a stratified epithelium on the external surface, an acellular collagenous Bowman's layer, a thick stroma, a Descemet's membrane, and a mono-layered endothelium on the internal surface (**Fig. 1**). These morphological features can vary among animals [2-4]. For example, the existence of a Bowman's layer in many species has been questioned. The corneal epithelium in humans is renewed by stem cells located at the periphery of the cornea, in a region known as the limbus (**Fig. 1**). The limbus anatomically starts where the corneal Bowman's layer ends [1]. The interest in the limbus has grown with increasing evidence for the limbal location of corneal epithelial stem cells in humans [5]. A failure of these stem cells results in the blinding disease of a limbal stem cell deficiency, which can be treated with limbal stem cell transplantation. The limbal stem cells are harbored in the basal layers of the limbal epithelium. The limbal stem cell niche, which is located superficially to the limbal stroma, maintains the limbal stem cells by providing vital bloodborne and stromal fibroblast-derived factors. Ultraviolet (UV) light affects the epithelium [6], but it can penetrate the corneal layers and lead to a dysfunction of the posterior monolayer of the eye, the endothelium [7]. Therefore, a protective mechanism must be identified to preserve the ocular surface that is in direct exposure to the UV light. Accordingly, the main aim of this research was to determine corneal and limbal morphological differences between species with respect to corneal epithelial maintenance, the limbal stem cells, and the limbal stem cell niche. The inter-species variability has not been described to the best of the authors' knowledge.

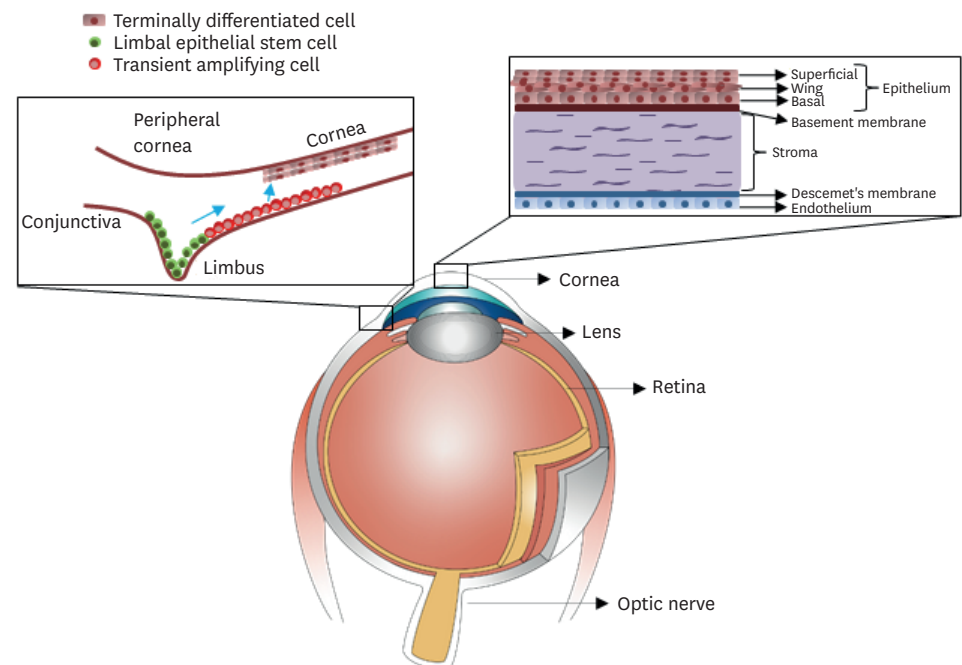


Fig. 1. Illustration of human eye globe with a cross-section of the human cornea and limbal region (not to scale). The cornea shows the epithelium, which is composed of squamous cells, wing cells, and columnar cells supported by Bowman's layer. The collagenous keratocytes contain stroma and a monolayer of endothelial cells that sits on the Descemet's membrane. The limbal region shows the reservoir of limbal stem cells and differentiated corneal epithelial cells.

MATERIALS AND METHODS

Ethical approval

Institutional (University of Liverpool) and Chester Zoo ethical approvals were obtained, and the Declaration of Helsinki was followed. Ocular globes from various species (based on availability) were obtained from Chester Zoological Garden, and human corneoscleral tissue was obtained from the UK Eye Banks after gaining the consent from the donor's next-of-kin.

Species studied

The following species were included in this study: human (*Homo sapiens*), Java sparrow (*Lonchura oryzivora*), spoonbill (*Platalea leucorodia*), macaw (*Anodorhynchus hyacinthinus*), penguin (*Spheniscus humboldti*), nutchuck frog (*Rana temporaria*), red panda (*Ailurus fulgens*), Doberman (*Canis lupus familiaris*), orangutan (*Pongo pygmaeus*), and horse (*Equus ferus caballus*). The species included in this study were based purely on their availability and donation from Chester Zoo.

Dissection and histology

The inclusion criteria were mainly based on the preserved quality of the globes. One globe was used from each species and was dissected to examine the cornea and limbus.

The tissue was fixed in 10% neutral buffered formalin at room temperature (RT) overnight. The tissue was then placed in a tissue-embedding cassette and processed. Each processed ocular globe was then embedded in paraffin wax using a mold. A microtome (Leica RM2235; Leica, Germany) was used to make 4 µm sections that were mounted on slides (Surgipath X-tra Adhesive Precleaned Microslides; Leica Biosystems, USA). Twenty slides were made from each embedded block. All slides were placed in an incubator (Leec, UK) at 37°C overnight.

Corneal thickness measurement

The corneal thickness was measured from at least three slides of the same species. All the measurements were obtained from a 20× magnification image with a 600-pixel resolution. A known distance of 100 microns was set to scale and analyze the images.

Hematoxylin and eosin (H&E) staining

Bowman's layer is the basement membrane of the corneal epithelial cells, which is an acellular non-regenerating layer. Removal of Bowman's layer could delay the subbasal nerve regeneration and subepithelial keratocyte density in humans, leading to faster stromal wound healing and anterior corneal transparency. The absence of a Bowman's layer could delay the stromal wound healing process or sub-basal epithelial reinnervation in humans. Therefore, identifying the presence of a Bowman's layer could help understand its role in different species. H&E stained stromal condensation beneath the epithelium was presumed to indicate a Bowman's layer.

The corneoscleral tissue sections from the different species were stained with H&E according to the following protocol. The slides were deparaffinized by immersion in the following for 5 min each to: xylene twice, 100% ethanol, 90% ethanol in distilled water, and 70% ethanol in distilled water, followed by running tap water for 30 sec. The slides were then immersed in Haematoxylin Gills III for 3 min, running tap water for 30 sec, 1% acid alcohol for 15 sec, running tap water for 5 min, alcoholic eosin for 5 min, and running tap water for 30 sec. The slides were immersed in 70% ethanol in distilled water for 30 sec, 90% ethanol in distilled water for 30 sec, and 100% ethanol for 30 sec. They were then finally immersed in xylene

twice for 1 min each. The slides were mounted in Pertex with a coverslip and allowed to dry overnight. All steps were performed at RT.

Periodic acid-Schiff (PAS) staining

The corneoscleral sections were stained using the following PAS's Reagent protocol. The tissue sections were deparaffinized by immersion in xylene 1 for 10 min and xylene 2 for 5 min, followed by 100% ethanol for 5 min, 90% ethanol in distilled water for 5 min, 70% ethanol in distilled water for 5 min, and hydrated in deionized water for 30 sec. The periodic acid solution was placed over the slides for 10 min. The slides were then rinsed with deionized water and immersed in Schiff's Reagent for 15 min, washed in running tap water for 5 min, and counterstained in Gill's Hematoxylin III for 15 sec and washed in running tap water. The slides were dehydrated by immersing them in 70% ethanol in distilled water for 30 sec, 90% ethanol in distilled water for 30 sec, and 100% ethanol, xylene 1, and xylene 2 for 1 min each. The slides were mounted in Pertex with a coverslip and allowed to dry overnight. All steps were performed at RT.

Imaging

All stained slides were visualized using a microscope (Nikon Eclipse Cli; Nikon, Japan). Photographs were taken with a color camera (Digital Sight DS-U3; Nikon) and analyzed using NIS Elements BR 4.13 software (Nikon).

Statistical analysis

A one-way analysis of variance followed by Tukey's multiple comparison test (unless otherwise specified) was used to determine statistically significant differences (GraphPad Prism 5, GraphPad Inc., USA; $p < 0.05$, $p < 0.01$, $p < 0.001$). The data are expressed as the median \pm 5th–95th percentile (unless otherwise specified).

RESULTS

Variations in total corneal and corneal epithelial thickness between species

The thickness of the corneas differs between species, but this difference could be related to the corneal stroma or the multilayer epithelial stratification. Thus, the overall corneal thickness and the corneal epithelial thickness were studied separately to identify the variation. The overall corneal and epithelial thickness varied among species. The orangutan had the thickest cornea ($730 \pm 59 \mu\text{m}$), and the Java sparrow had the thinnest ($166 \pm 5 \mu\text{m}$). The Java sparrow's corneal thickness varied significantly compared to orangutans ($p < 0.05$) and horses ($p < 0.05$) (**Fig. 2A**). Of the class Aves in this study, the penguin exhibited the thickest cornea (**Fig. 2A**), whereas the Java sparrow had the thinnest (**Fig. 2A**). The thinnest corneal epithelium was detected in the Java sparrow ($15 \mu\text{m}$). The horse had the thickest ($144 \mu\text{m}$) with 10 layers of epithelium (**Fig. 2B**). The epithelial thickness of the horse was significantly higher than the frog ($p < 0.05$) and Java sparrow ($p < 0.01$). The human epithelium was significantly thicker than the Java sparrow ($p < 0.05$). The corneal epithelium to total corneal thickness ratio was highest in horses and lowest in frogs (**Fig. 2C**).

The corneal epithelium of an orangutan was most similar to that of humans, composed of superficial non-keratinizing stratified squamous cells (stratum superficiale), wing cells (stratum intermedium), and columnar basal cells (stratum basale) (**Fig. 3A and B**). The Doberman (**Fig. 3C**) and macaw (**Fig. 3D**) also had similar corneal epithelial features. The

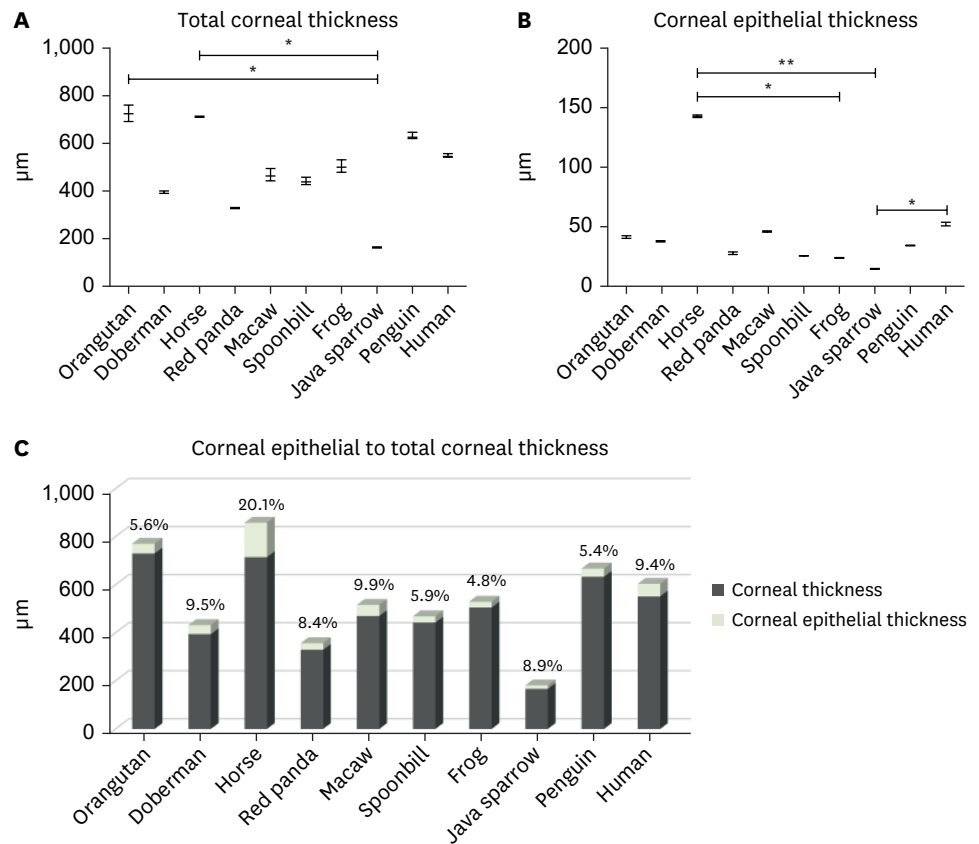


Fig. 2. Total corneal and epithelial thickness of different species. (A) Total corneal and (B) epithelial thickness of all the investigated species and (C) epithelial to the corneal thickness of all species included in this study ($n = 1$ [three readings] per species). One-way analysis of variance followed by a Tukey's multiple comparison test. * $p < 0.05$; ** $p < 0.01$.

penguin and Java sparrow expressed similar corneal epithelial features, with all layers composed of flattened squamous cells (**Fig. 3E and F**). A multi-layered epithelium was found in the horse cornea (**Fig. 3G**). The red panda, frog, and spoonbill corneas were composed of squamous epithelial cells superficially and cuboidal epithelial cells basally (**Fig. 3H-J**).

Variation in Bowman's layer and Descemet's membrane between species

The corneas from all species studied were composed of epithelium, stroma, and endothelium (**Fig. 3**), but the Bowman's layer varied between species. Only the orangutan and human corneas contained a Bowman's layer (**Fig. 3A and B**). The orangutan cornea was composed of a well-established Bowman's layer (**Fig. 3B**). The macaw cornea presented with increased stromal condensation beneath the epithelium that may be indicative of a rudimentary Bowman's layer (**Fig. 3D**). Other species from the class of Mammalia and all species from the class of Aves and Amphibia studied lacked Bowman's layer.

A homogeneous acellular membrane was found in the posterior cornea adjacent to the corneal endothelium of all species studied. This stained positively with PAS, confirming it as a Descemet's membrane.

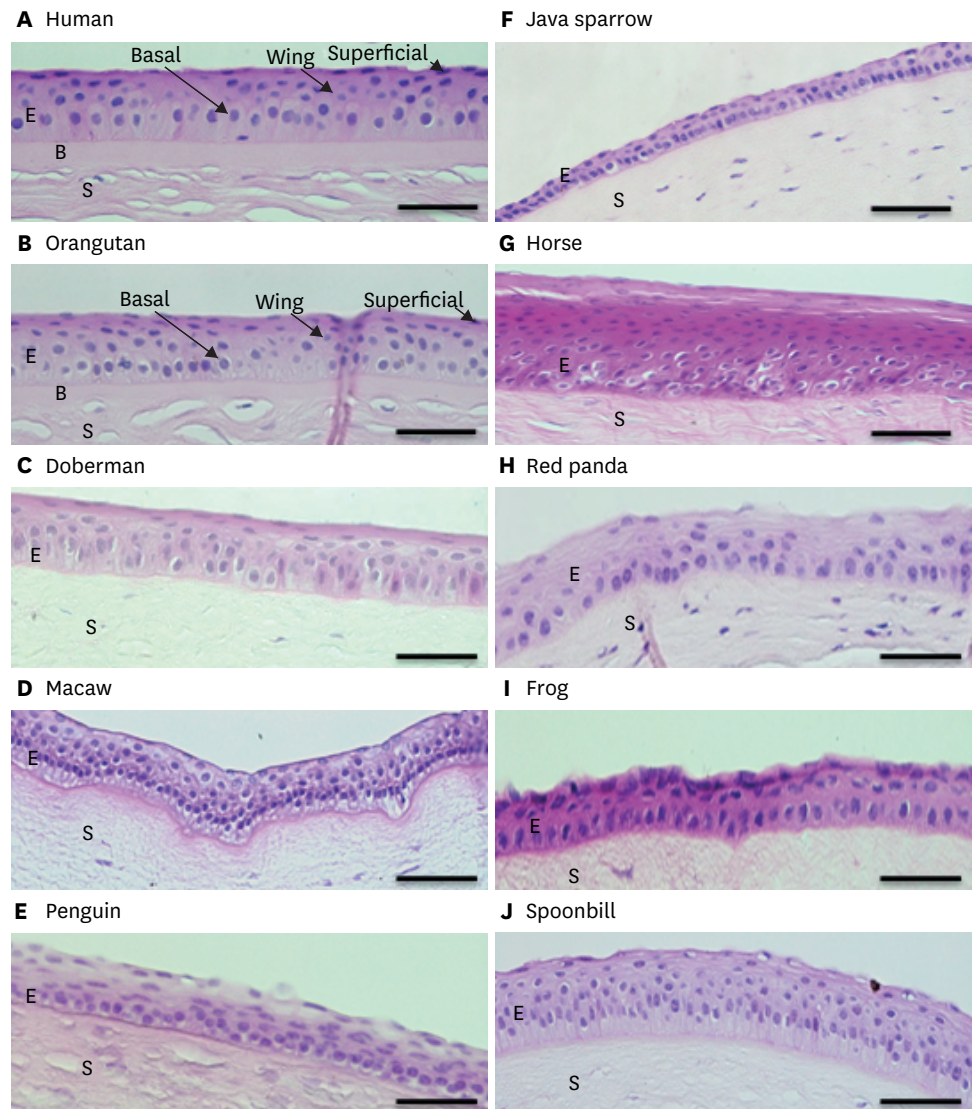


Fig. 3. Hematoxylin and eosin and periodic acid-Schiff staining of the corneoscleral rims showing corneal epithelial layers of (A) human, (B) orangutan, (C) Doberman, (D) macaw, (E) penguin, (F) java sparrow, (G) horse, (H) red panda, (I) frog, (J) spoonbill. Notice the black arrows in (A) and (B) showing the presence of Bowman's layer. Magnification: 40×; Scale bar = 50 μm.
E, epithelium; B, basement membrane; S, stroma.

Basal limbal epithelial cells show varying pigmentation between species, with the presence of a melanin capping over the nuclei

The posterior peripheral cornea houses a reservoir of limbal epithelial cells. These cells need to be protected to maintain corneal epithelial regeneration. The presence of a melanin capping on these cells could be advantageous for those animals that live in open grasslands or regions with a high UV index to protect these cells. The limbal region of the following four species exhibited pigmentation within the limbal epithelium: orangutan (**Fig. 4A**), horse (**Fig. 4B**), macaw (**Fig. 4C**), and red panda (**Fig. 4D**). In all these species, melanin-containing cells were found along the basal layers of the limbal epithelium, where the limbal stem cells putatively reside in humans. In the red panda, only the basal limbal epithelium cells showed pigmentation (**Fig. 4D**). Suprabasal limbal epithelial pigmentation was also seen in the

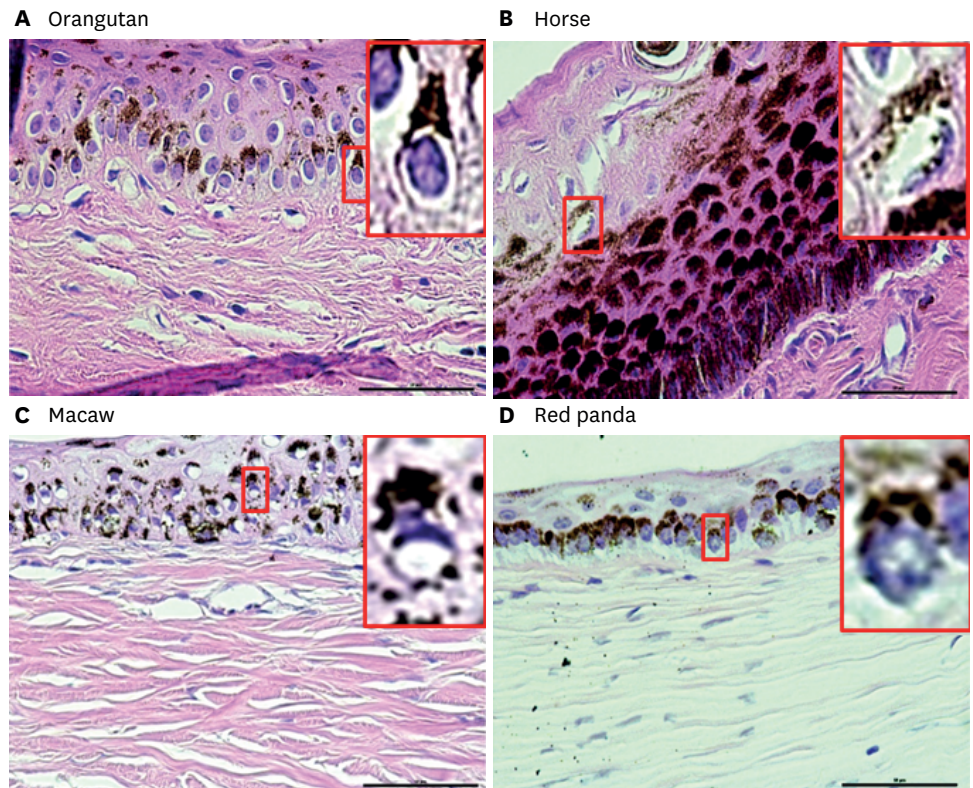


Fig. 4. Observation of supranuclear melanin caps. (A) Over each epithelial basal cell in an orangutan. (B) Pigmentation within the basal layer of a horse. Thick layer of pigmented granules is observed. (C) Macaw limbus epithelial pigmentation. (D) Red panda limbus epithelial pigmentation. The black arrows indicate supranuclear melanin caps on the apex of each cell. Magnification: 60 \times ; Scale bar = 50 μ m.

orangutan, horse, and macaw. The pigmentation observed in the horse limbal epithelium was the strongest, a finding possibly also correlated with the high number of epithelial cell layers compared to other species (**Fig. 4B**). Nevertheless, this is just an observation, and further investigation will be needed. The horse corneal epithelium also showed some degree of pigmentation. Interestingly, in these species, the melanin observed within the basal limbal epithelial cells was located in the form of a cap above the apical side of the nucleus (**Fig. 4**). All other species did not show melanin capping (**Supplementary Fig. 1A-E**).

DISCUSSION

The cornea, which is often recognized as the window to the world, is a vital boundary to any external insult. It also fulfills many unique functions, including light refraction [1]. In recent years, the corneal epithelium has been the center of interest because of the increasing knowledge of its stem cells and the development of replacement therapies for blinding corneal epithelial disease. Most studies used rodents as animal models for corneal epithelial stem cell research [8,9]. A comparative morphological study of the cornea and limbus in corneal epithelial maintenance and corneal epithelial stem cells of animals from different classes has not been performed previously, which was the aim of this research. This paper highlights the similarities and differences among ten different species from three different classes.

This study demonstrates the presence of a transparent cornea made up of an epithelium, stroma, underlying Descemet's membrane, and endothelium in all species studied. On the other hand, a Bowman's layer was only found in primates, similar to previous studies [2]. The macaw also presented with increased stromal condensation beneath the epithelium, which might indicate a rudimentary Bowman's Layer, as detected in a previous study [2]. In addition, the corneal epithelium thickness varied significantly between species. The corneal epithelium of horses consisted of 10 layers reflecting an overall thickness of 144 μm . The reason for this morphological difference was not explained in this study. On the other hand, other researchers in the past have hypothesized that the corneal thickness is related to the habitat of different species [10]. For example, horses are usually found in open grasslands, where exposure to rough environmental conditions is more likely. Similarly, a previous study identified a significantly thick corneal epithelium in camels that was attributed to the hot and dry habitat of the species [11]. The corneal thickness may vary between different cornea regions. Therefore, three readings were obtained from the center and at the periphery of all the species in this study to limit the error.

Regarding the limbal area, this study identified supranuclear melanin capsules in an area of the limbal epithelium where the limbal stem cells putatively reside. Specific limbal epithelial characterization was not performed because of a lack of resources (mainly, the number of corneal tissues from different species), which could limit this study showing melanin caps on basal limbal cells. On the other hand, anatomically, these cells resided precisely in the known limbal region. Therefore, the melanin caps are present on the basal limbal epithelial cells. This melanin capping was observed in the basal limbal epithelial cells of the following four species: horse, macaw, red panda, and orangutan. This finding of a pigmented limbus supports previous studies that observed a melanocyte population in the same area [11]. The limbal stem cells are essential for maintaining the corneal epithelium [1]. Some studies support the proposition of 'support' cells aiding the function of limbal stem cells. One study suggested that limbal epithelial stem cells and limbal melanocytes interact directly through N-cadherin homotypic cell adhesion and also proposed that melanocytes could play a role in maintaining limbal epithelial stem cells [12]. A different study has reported an interaction of melanocytes in the corneal limbus with K19(+) cells, a cytokeratin expressed by limbal basal cells [13,14]. This study showed melanocytes with dendritic processes surrounding epithelial cells forming supranuclear melanin caps. Similarly, this interspecies comparative study histologically showed the presence of supranuclear melanin caps in the basal epithelial layer where limbal stem cells are located. In the past, supranuclear melanin capping has been described as a process within the epidermis to reduce UV-induced production of DNA photoproducts [12]. UV light causes significant damage, including disruption of cellular functions, decreased cell motility, and genomic DNA mutations.

Most species in this study expressing supranuclear melanin caps originate from geographical areas with a high global UV-light index (orangutan, macaw, red panda). Therefore, it is understandable why they might have pigmentation acting as an anti-oxidant system to protect their stem cells. Pigmented granules found above each cell were observed at the apex of each cell, which is the direction of UV-light exposure. Orangutans are native to Indonesia and Malaysia and are found predominantly in the rainforest of Borneo. Borneo is located on the Equator itself and has a very high daily global solar UV index of 10.5–12.5. In addition, orangutans are the most arboreal out of all great apes. The atmospheric pressure decreases with increasing altitude, and less UV radiation is absorbed. Therefore, the function of the melanin granules within the orangutan limbal epithelium is possibly to protect its stem cells

from UV radiation. This is an example of a morphological interspecies variance due to the geographical distribution and habitat of the species. Similarly, the macaw is found in the Amazon rainforest of Venezuela, Colombia, and Brazil. These countries are situated in South America near the Equator, with a high global solar UV index. The red panda is a mammal found mainly in the mountains of Nepal and Burma. This species has a high-altitude forest habitat. The high UV exposure by this species is due mainly to the high altitude. The UV radiation increases by 10% to 12% for every 1,000 m increase in altitude. Knowledge of whether the same species living in different geographic areas present the same pigmentation levels or if this trait is inherited or acquired would be important to study in the future.

These results also showed high levels of pigmentation within the limbus of the horse (*Equus ferus caballus*). This study had several limitations, such as background knowledge of the horse breed. Some horse breeds are less pigmented than others. For example, Appaloosas and Paint horses express low pigmentation. They are also predisposed to a higher risk of squamous cell carcinoma of the periocular structures [15]. A similar association has been found for Hereford cattle, demonstrating that breeds with corneoscleral pigmentation are at lower risk [16]. Indeed, UV-induced genomic mutations leading to neoplasia occur most commonly in the limbal area [17].

The results of this comparative study show clear interspecies variations through histological methods. The orangutan's corneal structure has similar layers and thicknesses to humans. A clear Bowman's layer was only found in primates. On the other hand, all species presented with a Descemet's membrane, albeit with varying thickness. In addition, supranuclear melanin capping occurred in the basal layer of the limbus epithelium of four species (orangutan, macaw, red panda, and horse). The melanin granules covered the apex of each limbal stem cell. This pigmentation was only found in species living in areas with high UV exposure or open surrounding. Thus, there is a possible relationship between limbal stem cell melanin capping as a protective mechanism against ultra-violet radiation. Although the study was limited by the number of tissues analyzed, the results indicate a proof-of-concept requiring further study to determine if melanin is inherited or acquired based on the geographic origin.

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SUPPLEMENTARY MATERIAL

Supplementary Fig. 1

Hematoxylin and eosin and periodic acid-Schiff staining of the corneoscleral rims of other species not showing melanin caps at their respective magnifications. (A) Java sparrow: 20×; (B) Frog: 20×; (C) Doberman: 40×; (D) Penguin: 40×; (E) Spoonbill: 20× and (F) Horse: 20×. The black arrow on (F) indicates a positive Descemet's membrane.

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