The Role of the Ocular Lids of the Black-Winged Kite, Elanus caeruleus , in the Immune Protection of the Eye

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Abstract

The aim of the study was to describe the morphological features and microstructure of the upper, lower, and third eyelids of black-winged kite, *Elanus caeruleus*, as well as characterize the organized lymphoid follicles and lymphocytes in the eyelid's mucosa, and to illustrate the importance of the eye adnexa in the eye's immune protection. The black-winged kite has large forward-facing eyes placed under a bony shelf (lacrimal process) that shaded them. Both eyelids have thick and pigmented edges and bear two rows of long and finely modified filoplume feathers that increase at the anterior canthus. It was found that the lower eyelid appears longer and thinner than the upper one, as well as having sparse feathers on the skin surface of the lower eyelid but lack on the upper. The third eyelid is a white opaque membrane moving obliquely over the cornea surface. The melanocytes appear in the stratum basal of upper and lower eyelids and the Langerhans cells were observed within the layers of stratum spinosum, near the feather follicle and around the blood vessel. Aggregations of lymphatic cells were present under the conjunctival epithelium within the stroma of the lower eyelid (in the orbital zone near the tarsal plate), while absent in the upper eyelid and nictitating membrane. The present study revealed that many high endothelial venues (HEV) are distributed along the lower eyelid and increase in the palpebral marginalis, while in the upper eyelid, it is restricted in the marginal region. The density of goblet cells on the conjunctiva surface of the upper eyelid and the nictitating membrane is higher than that of the lower eyelid. The leading edge and bulbi surface of the third eyelid reveal the irregular surface of the apical cell, with many cilia having variable amounts of secretory vesicles as shown by TEM.

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Abstract

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Keywords:

Eyelids, Nictitating membrane, Conjunctiva-associated lymphoid tissue (CALT)**,** Immune protection.

Introduction

The avian eye's greater capabilities than the human eyes are attributed to the birds' unique lifestyle and environments, as well as to physical activities like flight that require excellent vision. The ecomorphological design of the vertebrate eye reflects the characteristics of the species' environment and lifestyle. Although the morphological aspects of the eye are similar for all vertebrates, its microstructural and molecular features vary among species from different ecological niches. The accessory organs of the eye (eye adnexa) are important for proper functioning of the eye (Bayon et al. 2007). They include the eyelids (upper, lower, and third eyelids), conjunctiva-associated lymphoid tissue (CALT), the Harderian gland (HG), and the lacrimal gland (LG) (Klec´kowska-Nawrot et al. 2015). These structures mainly moisturize the eye and supply the superior and inferior conjunctival sacs with nutrients and antibodies to maintain their health (Dartt, 2009; Maggs, 2013; Jochems & Phillips, 2015; Klećkowska-Nawrot et al. 2016).

Recently, it was recorded that many viruses replicate in the feather pulp and/or the feather follicle epithelium, such as those found on the eyelids (Takashi et al. 2021), for example, Marek's disease virus (MDV), avian leukosis virus, and chicken infectious anemia virus. Each one of these diseases may cause a partial loss of vision and have far-reaching effects, and the compensatory use of other senses (such as auditory and olfactory sensory perceptions) is typically insufficient (Ahmed et al. 2016)

Ornitho-ophthalmology has played a significant role in this field with very few exceptions e.g., pathogens transmitted via the germline, the animal pathogens enter the host by breaching the barrier between the external and internal milieus. This barrier consists of specialized tissues characterized by two main components: an externally located epithelium and the underlying connective tissue (Korbel, 2000; Korbel & Bohnet, 2007). So, to prevent the entry of pathogens through the mucosal tissues, a wide variety of protective mechanisms have evolved. These range from barrier functions (e.g., keratinized skin) to highly specialized immune cells (e.g., Langerhans cells in the skin) and the organized lymphoid structures in the mucous membrane. Obviously, the epithelial cells of the mucosal surfaces are able to sense pathogens and actively shape the response of the immune cells underneath (Alan & Athena, 2019).

For many years, research in mucosal immunology has focused on the question of how these cells become activated and interact to protect the host from invasion and dissemination of pathogenic microorganisms (Yasmine & Timothy, 2014). Under physiological conditions, the mucosal immune system has to remain tolerant of the microbiome to avoid chronic mucosal inflammation, while at the same time being able to respond quickly and appropriately to pathogenic challenges. This explains the great importance of the eyelids in protecting the eye from diseases and that they are not only responsible for controlling the amount

of light and moisturizing the eye but also play an important role in protecting the eye from diseases (Knop N & Knop E 2005 a &b).

The present authors selected a common wild bird species in my country, the black winged kite to investigate the morphological features and microstructure as well as demonstrate the lymphoid follicles and lymphocytes to illustrate the importance of the characterization of the eye adnexa in the protecting of the eye from any diseases.

MATERIAL AND METHODS

Ten adult specimens of the black-winged kite, *Elanus caeruleus,*were brought to the comparative anatomy of Vertebrate Laboratory, Zoology Department, Faculty of Science, Assiut University, in a condition of good health, and cared for according to the guidelines of the Research Ethics Committee, Assiut University (under the no. 06/20230099).

Anatomical study

The heads of five specimens were fixed in 10% formalin for two weeks and then stored in 2% phenoxy-ethanol for long-term preservation. Photographs were taken using a Toup camp XCAM full HD camera. The present study followed Nomina Anatomica Veterinaria (Rio ,2017) for anatomical terminology of the eyelid's musculature system.

Histological study

Three specimens of eyelids were fixed in 10% neutral formalin for three days, then put in 70% ethyl alcohol for two days, after which they were dehydrated through a series of ethyl alcohols, cleared in methyl benzoate for three days, embedded in paraffin wax, sectioned serially (5μm) and then sections were stained with Hematoxylin and Eosin, Masson's trichromic stain, and periodic acid-Schiff (PAS) (Drury & Wallington, 1980).

Scanning electron microscopy study

The eyelids of two specimens were cut into small pieces and directly fixed in 5% glutaraldehyde in a cacodylate buffer for 48 h at 4°C and washed in three changes of 0.1% cacodylate buffer, and then, the specimens were post-fixed in a cacodylate-buffered solution of 1% osmium tetroxide for 2 h at 37°C. The specimens were washed in the same buffer three times, dehydrated through an ascending series of ethyl alcohol, and then infiltrated with amyl acetate for 3 two days. The drying of the specimens was accomplished by the critical point drying using liquid carbon dioxide, mounted and sputter-coated with gold. The specimens were examined on a Jeol scanning electron microscope (JS M-5400IV) at 15 kv.

Semithin sections and transmission electron microscopy examination

For transmission electron microscopic investigation (TEM), parts from the eyelids were cut into small pieces (1 mm each) and were fixed in a cacodylate-buffered solution of 5% glutaraldehyde for 2 h, then washed several times in the same buffer for 1hr at pH = 7.2, and then, the specimens were post-fixed in a cacodylate buffer 1% osmium tetraoxide for 2 h at 4°C. Specimens were washed several times in the second step, followed by dehydration in a graded series of alcohol. The specimens were embedded in epoxy resin; they were treated for semi-thin sectioning at 1 mm thickness and stained with toluidine blue for light microscopic examination. Photographs were taken with an Olympus camera model DP74 connected with an Olympus microscope model BX43.

Ultrathin sections of 70 nm thickness were cut by Ultratome VRV (LKB, Bromma, Sweden), and stained with uranyl acetate and lead citrate (Reynolds, 1963), then were examined by JEOL 100CX II transmission electron microscope at the Electron Microscopy Unit of Assiut University, Egypt.

Immunohistochemical study

For immunohistochemistry, tissue sections were processed according to (Ramadan et al. 2018). We stained the tissue sections with the following primary antibodies: anti-CD3, anti- CD20, anti-CD138 and anti-CD1a.

Results

Morphological investigation of the upper and lower eyelids of*Elanus caeruleus*

The black-winged kite, *Elanus caeruleus* has large forward-facing eyes placed under a bony shelf (lacrimal process) that shades them (Fig.1a). The skin covering the lacrimal process carries regular rows of feathers, like a brow, which appears as a canopy to cover the upper eyelid (Fig.1 b). This bird has un-drooping eyelids, and the lower eyelid appears more extensive and larger than the upper eyelid (Fig.1 b).

The palpebral margin of each eyelid is pigmented at variable degrees and puckered into bumps that increase toward the anterior canthus (Fig.1d). Near the margin of each eyelid, there are two rows of long and finely modified filoplume feathers as eyelashes (Fig.1c). Meanwhile, on the lower eyelid skin, filoplume feathers spar, while no feathers appear on the upper eyelid. Furthermore, a collection of copious plumage exists next to the anterior canthus of the eye (Fig.1b).,

Scanning electron microscopy investigation of the upper and lower eyelids of the *Elanus caeruleus* revealed the presence of heavy detached keratin and modified filoplume feathers at the ridge of the palpebral margin of each lid (Fig.2 a& b). The detached keratin disappeared at the mucocutaneous junction (MCJ) (Fig.3a), and the intercellular borders are clearly observed, as well as many scattered pores appearing on the conjunctival surface (Fig.3 b& c). In the lower eyelid, these pores are restricted \soutin to the tarsal region, and increase in size and density in its orbital region (Fig.3d). Furthermore, the microfold cells (M cell) were noticed on the surface of the two eyelids (Fig.3c) and Langerhans cells were observed underlying the basal lamina protruding their dendrites and connected with the collagen fibers (Fig.2c). Moreover, macrophage cell with filamentary pseudopods in between the collagen bundles (Fig.2d).

Histological investigation of the upper and lower eyelids of*Elanus caeruleus*

The upper and lower eyelids of black-winged kite *Elanus caeruleus* have an outer skin and an inner conjunctival surface. Histologically, the conjunctival surface of the upper eyelid is defined into two regions, the marginal and orbital regions (fig.4), While in the lower eyelid, it is composed of the marginal, tarsal, and orbital regions (Fig.5).

The skin of both eyelids is composed of keratinized stratified squamous epithelium. The skin of the upper lid consists of six to seven nucleated cell layers (Fig.4a), while it consists of three to four nucleated- cell layers in the lower eyelid (Fig.4b, 5a). These epithelial layers increase gradually towards the palpebral margin to become ten nucleated cell layers in both eyelids (Fig.5a). The skin of both eyelids is covered by a thin layer of keratin, which is lacking at the MCJ and the conjunctival surface (Fig.4b&c, 5b&c).

Semi-thin sections of the skin of both eyelids of *Elanus caeruleus* revealed the details of different layers. The stratum basal, which is composed of cuboidal cells with rounded nuclei, contains melanocytes with spherical and oval nuclei and numerous melanin granules in their cytoplasm; and the stratum spinosum, where the keratinocytes have rounded nuclei and adhere to each other by desmosomes. Within these layers, it was noticed that the Langerhans cells have light cytoplasm, lack melanin granules, and their nuclei are kidney-shaped or lobulated. The stratum granulosum forms two layers of polyhedral keratinocyte cells, which are flatter and more irregular in shape than those in the stratum spinosum. Finally, the stratum corneum which lacks nuclei is formed of wavy keratin (Fig.6a&c).

The conjunctival surface of the upper eyelid in the orbital region consists of stratified cuboidal epithelium bearing a short cytoplasmic extension at the apical margin of cells (Figs.4d). This epithelium toward the fornix conjunctiva palpebral converted to stratified columnar type and appeared as a cells-cluster with small pockets/space in between them. These clusters lack the cytoplasmic extensions and have irregular surfaces (Figs.4e). In addition, the epithelium of conjunctiva containing numerous goblet cells which exhibit purple color with PAS reaction, the goblet cells increase in density toward the fornix conjunctiva palpebral.

(Fig.4e). Meanwhile, in the lower eyelid, the conjunctival at the MCJ and the marginal region, is composed of nine layers of non-keratinized stratified squamous epithelium with scattered single mucous cells (Fig.5c&d). Whereas, only three to five layers of stratified cuboidal epithelium were seen in the tarsal and orbital region, respectively (Fig.5e). The goblet cells scattered in the lower eyelid of the orbital region and increased toward the fornix (Fig.5e).

Semi-thin sections of the conjunctival surface of both eyelids of*Elanus caeruleus* revealed the presence of M like cells in the superficial layer. They are characterized by euchromatic nuclei, pale cytoplasm filled with vesicles of various sizes, and surrounded by intraepithelial lymphocytes (IEL) Moreover, neighboring enterocytes and goblet cells with pale cytoplasm were observed (Fig.6 b & d).

The stroma of both eyelids of*Elanus caeruleus* contained a scattered network of collagen fibers and contained many feather follicles in the lower lid which were not seen in the upper eyelid (Figs. 4b and 5c& e). Moreover, a sparse number of histiocytes, blood vessels and few heavy endothelial venues (HEV) were observed (Fig.4c&5e). The stroma of the marginal region of both eyelids is highly vascularized (Fig.4b& 5b). Numerous diffuse lymphocytes were scattered in the connective tissue of the lower eyelid beneath the orbital region of the conjunctival epithelium, which became more extensive near the tarsal plate (Fig. 5e). Numerous blood vessels and heavy endothelial venule (HEV) were observed around the inferior tarsal plate (Fig.5b, d).

Ultrastructure investigation of the upper and lower eyelids of*Elanus caeruleus*

Transmission electron microscopical study of the eyelids skin of*Elanus caeruleus* revealed melanocytes of spherical to oval shape and are located mainly in the basal layer of the epidermis. Moreover, they have an oval nucleus with clumps of condensed chromatin. The cytoplasm of the melanocytes has numerous melanin granules and the cytoplasmic processes of melanocytes were also clearly observed (Fig.7a &b).

The keratinocytes in stratum spinosum are characterized by a large rounded euchromatic nucleus. They are tightly connected by numerous tonofilaments (keratin filaments) crossing the intercellular spaces and extending into junctions between the cells (Fig.7a &b).

The Langerhans cells are non-pigmented, and found in the basal and spinosum layers. They are characterized by pale cytoplasm, deeply indented and heterochromatic nucleus and the presence of specific Langerhans granules. In addition, free ribosomes, mitochondria and some membranous vesicles of variable size (Fig. 7a & c). The stratum corneum constitutes the superficial layer, which lacks nuclei and is formed of wavy keratin (fig .7a)

The ultrastructure of the conjunctival epithelium of the lower eyelid revealed irregular cytoplasmic extensions emerging from the cell surface. Moreover, the epithelial cell contains a round and euchromatic nucleus and is enriched with smooth endoplasmic reticulum, few mitochondria and numerous membranous vesicles of varying size (Fig. 7d).

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Morphological investigation of the nictitating membrane of *Elanus caeruleus*

The nictitating membrane of *Elanus caeruleus* is a white opaque membrane, have palpebral and bulbar surfaces (Fig.8a). Anatomical and scanning electron microscopy of the nictitating membrane revealed several folds on their surfaces (Figs.8 a& c). the irregular surface of the free margin has a few shallow pores (Fig.8b). Furthermore, moderate-density cytoplasmic extension appeared in the apical cells on the bulbar surface (Fig. 8), which disappears towards the fornix conjunctiva bulbi and the intercellular border of the bulbar surface (Fig.8e). Many pores scattered between the folds of the bulbar surface (Fig.8e).

The laboratory observation of the movement of the eyelids of*Elanus caeruleus* showed that the nictitating membrane slips rapidly in an oblique direction from the dorsonasal angle toward the ventrotemporal direction (Fig.9).

Histological investigation of the nictitating membrane of*Elanus caeruleus*

The nictitating membrane of *Elanus caeruleus* is divided into two regions; the head and body. Further, the head of the membrane can be distinguished into two parts: the plica marginalis (free margin) externally and the leading edge internally (Fig.10 a& b), and both are covered by stratified epithelium (Fig. 10 b). This epithelium changes into stratified cuboidal epithelium to form the leading edge (Fig. 10 b). This stratified cuboidal epithelium has short cytoplasmic extensions (Fig. 10 b), which decrease gradually and then disappear toward the fornix conjunctiva bulbi (Fig. 10 c). Numerous pigmented granules are interspersed in the connective tissue of the plica (Fig. 10 b). The body of the nictitating membrane possesses two surfaces: the palpebral and bulbar surfaces. Both surfaces are covered by stratified cuboidal epithelium with an irregular apical membrane (Fig. 10 c). while This epithelium changes into the stratified columnar type containing goblet cells toward the fornix conjunctiva bulbi (Fig.10 d).

The high magnification of the epithelium of the bulbar surface of the nictitating membrane of *Elanus caeruleus* revealed four nucleated cell layers; the stratum basal has cells with irregular basal lamina forming basal finger-like fine protrusions (Fig. 11 b). The apical membrane of the superficial cells located between the folds has a short cytoplasmic extension. Moreover, there are numerous scattered goblet cells, which increase in the fornix conjunctiva bulbi (Fig. 11 b). The palpebral surface is composed of six nucleated cell layers. The superficial cells have pale cytoplasm and irregular apical membrane (Fig. 11 a)

Ultrastructure investigation of the nictitating membrane of*Elanus caeruleus*

The ultrastructure of the nictitating membrane of *Elanus caeruleus* revealed the flattened cells of the palpebral surface appear loosely connected with each other by complex interdigitations of the cell membranes. Furthermore, the cytoplasm contains many cytoplasmic vacuoles of varying size (Fig. 12 a).

The Immature M cells observed in the bulbar surface are lighter compared to the neighboring enterocytes and abundant various sizes of vacuoles in the apical cytoplasm. M cells exhibited shorter and irregular microvilli on their apical membrane compared to the neighboring enterocytes. In addition, goblet cell with free edge (Fig. 12 b & c) and enterocyte-like columnar cells were observed (Fig. 12 b & c)

Immunohistochemical investigation of the upper, lower and nictitating membrane of *Elanus caeruleus*

Immunohistochemical study to the upper and lower eyelids revealed that the CD1a-positive Langerhans cells of skin are distributed in the stratum basal more than the stratum spinosum of the squamous epithelium. As well as, it appears in the stroma around the blood vessels, HEV and around and inside the feather follicle. Moreover, no Langerhans cells were observed near the conjunctiva surface.

In addition, there is a varying concentration and un-equal distribution of lymphocytes within the CALT. Where, the B cells which cluster under the basement membrane were higher number than T cells which scattered close to blood vessels. While, the plasma cells were the fewest and randomly distributed within the CALT and it increase around HEV.

In the third eyelid, population of CD20+(B cell), CD3+(T cell) and CD1a+ (Langerhans cells) increase in density around the blood vessels, where the highest concentration was to T cells, while, the lowest population of the plasma cells diffuse as singular cells between the two surface of the membrane.

Discussion

The black-winged kite (*Elanus caeruleus*), also known as the black-shouldered kite, is a small diurnal prey-predator bird. In 1987, Jaksic studied the behavior of this bird and concluded that the changes in food requirements (breeding and nonbreeding) were met entirely by changes in hunting yield. The visual acuity of these kites is one of the important tools in the hunting process (Potier, 2020).

The present study observed a modified filoplume feathers spars on the skin of the lower eyelid and a collection of copious plumage at the anterior side of the eye, similar results had been observed by (Mahmoud et al.

2022) in Little Owl and (Jochems & Phillips, 2015) in Barred Owl. Anatomically, the feather near the eyelid is characterized by the absence of a vane. This type of modification protects the eye from ultraviolet radiation injury and prevents the adhesion of food and dust particles during feeding and fighting. (Mendelsohn,1983) pointed out that soft feathers can adhere with some grass seeds, whilst a long and wide bony shelf protected the upper eyelid which lack feather. Also, the unfeathered areas have a much denser supply of blood vessels than skin in feathered areas (Negro et al. 2006). This explains the high vascularization in the thick skin of the upper eyelid and the palpebral margins of both eyelids of the black-winged kite.

Elanus caeruleus possesses a thin lower eyelid with feathers, and the movement observation recorded that the lower lid moved more than the upper. Those observations explain the role of the lower eyelid in the protection of the eye against ultraviolet radiation injury when closed. (Knop et al. 2010 and 2011) pointed out the feather follicles provide the epithelial stem cells that help repair wounds, as well as, the thick skin in the marginal region provides protection to areas that experience more friction and abrasion.

The present study demonstrated the presence of Langerhans cells in the skin of the upper and lower eyelids. Langerhans cell in *Elanus caeruleus* increases in number in the lid margin. Langerhans cells serve as "sentinels" at the interface of the external environment and the immune system, and it bridges aspects of the innate (skin) and adaptive (T cells) immune responses (Neagu et al. 2022). They are stellate cells that protrude their dendrites via tight junctions toward the stratum corneum and as such can probe for antigens across several layers of the epidermis without disrupting the permeability barrier (Deckers et al. 2018).

The present study observed the presence of M like cells in the different parts along the conjunctiva surface of both eyelids of *Elanus caeruleus* and the nictitating membrane and associated with the surfaces facing the eye fornices. Similar results were recorded in fowl and turkey, which also were similar in many respects to those found in mammals (Maxwell et al. 1986). (Fix & Arp, 1989) referred to the association of M-like cells with the underlying lymphoid tissue in the turkey CALT epithelium. (Oya et al. 2021) also observed the presence of the M like cell in the tear duct-associated lymphoid tissue (TALT) and suggested that it may contribute to immune surveillance in the eye region because of their uptake capacity of the luminal nanoparticles.

The MCJ of the marginal region of the lower eyelid of *Elanus caeruleus* is composed of nine layers of non-keratinized stratified squamous epithelium with scattered single mucous cells which does not found in the margin of the upper eyelid. (Knop et al. 2011) mentioned the presence of goblet cells in human lid margin the goblet cells regularly occurred either as single cells or arranged into groups. The goblet cells were also observed in the conjunctiva of the upper and lower eyelid and the nictitating membrane of *Elanus caeruleus.* The goblet cells are essential components of the conjunctival mucosal immune system and they produce immune-regulatory factors, such as TGF- β215 and retinoic acid (RA) (Xiao et al. 2018; Gipson, 2016; Gipson et al. 2014). They mentioned the function of the goblet cell, whereby mucin maintains ocular surface hydration, stabilizes tears and removes pathogens and debris.

The conjunctival epithelium differed between the non-lymphoid and lymphoid regions, where the non-lymphoid conjunctiva is covered by a stratified columnar epithelium containing goblet cells, while in lymphoid regions; the epithelium does not contain goblet cells, and aggregation of the solitary lymphoid follicles (Kageyama et al. 2006; Bayraktaroglu et al. 2011). The present histological and scanning electron microscopy investigations of *Elanus caeruleus* showed the different distributions of goblet cells and of lymphoid follicles aggregations in the eyelids. Numerous goblet cells varying in size are localized in the conjunctival epithelium of the lower eyelid close contact with lymphatic aggregation near the tarsal plate

Some animals were similar to *Elanus caeruleus,* such as*Chinchilla lanigera* (Voigt et al. 2012) and guinea pigs (Gasser et al. 2011), where numerous goblet cells appeared in the bulbar and palpebral conjunctiva zone, and occasional goblet cells in lymphoid regions in the upper and lower eyelid.

The distribution of the lymphoid follicles differs between different families and species such as in chicken, it was observed the lymphoid follicles in the upper eyelid are much smaller and are located near the lacrimal ducts (Van Ginkel et al. 2012). Whereas, (Franklin & Remus, 1984) indicated the presence of diffuse

lymphocytes in the rabbit eye. (Klećkowska-Nawrot, Nowaczyk, et al. 2016) observed that the human conjunctiva contains lymphoid follicles in both the lower and upper eyelid, on the other side, rodents (such as rats and mice) do not contain lymphoid follicles under physiological conditions, and have very few diffusely interspersed lymphoid cells.

The lymphatic cell aggregation observed in *Elanus caeruleus* were present only in the lower eyelid and were small compared with those in other animal species studied by (Chodosh et al. 1998). We noticed them under the conjunctival epithelium near the tarsal plate, as well as, the Phoenicopterimorhae and Procellariimorphae, examined by (Klećkowska-Nawrot, Nowaczyk, et al. 2017), whereas in the Strigimorphae solitary lymphoid follicles were located under the tarsal plate.

The lymphoid follicles contain a heterogeneous population of lymphocytes within the conjunctival folds and fissures of the avian lower eyelid (Fix & Arp, 1991). In the adult African black ostriches, the aggregation of lymphoid follicles were observed mostly in the lower eyelid, however, in the upper eyelid, they were limited to the nasolacrimal punctum (Bayraktaroglu et al. 2011).

The present study revealed that the conjunctiva-associated lymphoid tissue in the eyelid of *Elanus caeruleus* consists of the intraepithelial lymphocytes, subepithelial lymphoid cells and blood vessels. The diffuse CALT was also located within the conjunctival folds under the conjunctival epithelium, similar results were observed in ostriches by (Klećkowska-Nawrot et al. 2016). Lymphocytes from the bloodstream pass to the lymphoid tissue principally via specialized high endothelial venules (HEV).

However, the present investigations revealed that the diffuse lymphoid tissue of lymphocytes (predominantly T cells), B lymphocytes and plasma cells formed a thin layer in the lamina propria of the conjunctiva, and around the high endothelial venules (HEV) distributed along the lower eyelid and increased in the palpebral margins while in the upper eyelid restricted in the marginal region. In humans, HEV are located in diffuse lymphocytes and lymphoid follicles. However, in birds (Bilgorajska goose), the diffuse lymphocytes are located both within and around the HEV (Klećkowska-Nawrot, Nowaczyk, et al. 2016; Knop & Knop, 2003(. From the results that mentioned above, the structure of the lower eyelid of black kite enhances ocular immunity than the upper eyelid.

Conclusion

The differences in the morphology of the upper, lower and third eyelid seem to strongly correlate with the lifestyle of the birds reflecting an adaptation to the habitat and feeding. The structure of the skin and conjunctiva of both upper and lower eyelids contains many defensive immune cells that maintain the safety of the eye and the hydration moreover, the lower eyelid's CALT constitutes the majority of CALT tissue in *Elanus caeruleus* , and is considered part of the mucosal immune system.

Figure legends

FIGURE 1: Photomacrograph of the eye of *Elanus caeruleus,*showing: (a) forward-facing eye placed under a lacrimal process (LP). (b) regular row of feathers (arrow) on the lacrimal process (LP), the lower eyelid (LL) more extensive and larger than the upper eyelid (UL) and filoplume feather spars on the skin surface of the lower eyelid (black arrowhead). (C) two rows of feather, like eyelashes on palpebral margin (zigzag arrow). (d) the palpebral margin of each eyelid puckered into bumps toward the medial canthus (double arrows).

FIGURE 2. Scanning electron micrograph eyelids of *Elanus caeruleus* , showing: (a) the detached keratin in the skin surface of the eyelid (arrowhead). (b) a modified filoplume feather at the ridge of the palpebral margin (arrow). (c) Langerhans cells (L) protrude their dendrites (black arrowheads) and connected with the collagen fiber (c). (d) Macrophage (M) with filamentous pseudopods (zigzag arrow) in between the collagen bundles(c).

FIGURE 3. Scanning electron micrograph eyelids of *Elanus caeruleus,* showing: (a) skin surface (Ss) and conjunctival surface (cs) and the disappearance of keratin (arrowhead) at the mucocutaneous junction (MCJ). (b) scattered pores on the conjunctival surface of the upper eyelid in the orbital region (zigzag arrow). (c

and d) pores in the conjunctival surface of the lower eyelid at the tarsal region (c) which increase in size and density in the orbital region. (d) moreover, sparse secretion (black arrow). in addition, M cell (purple arrow).

FIGURE 4. Photomicrograph of a transverse section of the upper eyelid of, *Elanus caeruleus,* showing: (a) the main layers of the skin surface (Ss) which covers with wavy keratin (black arrow) (scale bar, 50 µm). (b) the disappearance of keratin at (MCJ) in the palpebral margin (Pm), the collagen fibers within the stroma (St) and High vascularization of the margin (red arrow), (scale bar,50 µm). (c) the conjunctival surface (Cs) in the marginal region and heavy endothelial venules (double arrow). (d) conjunctival surface of the upper eyelid (Cs) in the orbital region is composed of stratified columnar epithelium carries short cytoplasmic extension in the apical cell (arrowhead) (scale bar, 50 µm). (e) in the fornix conjunctival palpebral, the epithelium appears as a cluster of cells with space in between them (zigzag arrow) and the submucosa is rich in collagen fibers (green color), (Masson's trichrome, upper left of (e) by PAS scale bar, 50 µm).

FIGURE 5. Photomicrograph of a transverse section of the lower eyelid of*Elanus caeruleus,* showing: (a) the skin surface coved by a thin layer of keratin (zigzag arrow) blood vessel (blue arrow) and collagen fiber in the stroma (st) .(b) the change in the epithelium thickness and keratinization along the lower eyelid and disappearance of keratin at MCJ and conjunctiva in the marginal region (double arrowhead). (c) single mucus cell within the epithelium in the marginal region (red arrow). (d) the conjunctiva in the tarsal region invaded by goblet cells (black arrow) and HEV (double zigzag arrow). (e) conjunctiva in orbital region and aggregation of lymphoid cell (L) goblet cells (black arrow). (a, c, d e by Masson's trichromic upper right by PAS, b by H & E, scale bar,50 µm).

FIGURE 6. Photomicrograph of a semithin section of the upper and lower eyelid of *Elanus caeruleus* , showing (a). different skin layers of the upper eyelid. (b) conjunctival surface of the upper eyelid (Cs). (c) different skin layers of the lower eyelid the: stratum basal contains melanocytes (red arrowhead), the keratinocytes adhere to each other by desmosomes (white arrow), Langerhans cells (black arrowhead). the stratum corneum is formed of wavy keratin (red arrow). (d) conjunctival surface of the lower eyelid: which contain goblet cell (double arrowhead), M cell in the superficial layer (zigzag arrow) and enterocytes (double arrow). (toluidine blue, scale bar,100, µm).

FIGURE 7. Transmission electron micrograph of the upper and lower eyelid of *Elanus caeruleus*showing (a & b) skin layer of the eyelid: basal lamina (B), melanocyte (M), keratinocyte (K), Langerhans cell located basally (L) and tonofilaments (keratin filaments) (zigzag arrow) and the stratum corneum without nucleus (C) and keratin (double arrowheads) cytoplasmic process of melanocyte (white arrow). (c) Langerhans cell contain specific granule (blue arrowhead), mitochondria (black arrowhead), and membranous vesicles (zigzag arrow). (d) the epithelium of lower lid conjunctiva with short cytoplasmic extension (black arrow) smooth endoplasmic reticulum (s), mitochondria (black arrowhead), and numerous membranous vesicles (red zigzag arrow) (Mic. Mag. X 7200).

FIGURE 8. Gross and scanning electron micrograph of the nictitating membrane of *Elanus caeruleus,* showing:(a) Photomacrograph of the nictitating membrane showing head of the membrane (H), body (B) free margin (FM) upper lid (UL) lower lid (LL) and fornix conjunctiva palpebral (f.c.p). (b) flattened cells in the free margin (zigzag arrow). (c) folds in bulbar surface. (d) the cytoplasmic extension on the bulbar surface (red arrows). (e) the bulbar surface of the membrane (arrow) becomes more flattened towards the fornix conjunctiva, interspersed with scattered pores and intercellular border (red arrowhead).

FIGURE 9. Photo of a sequence of video recording of the movement of the nictitating membrane of *Elanus caeruleus* showing this membrane slips rapidly and obliquely across the front of the eye from a fixed point towards the ventrotemporal direction

FIGURE 10. Photomicrograph of a transverse section of the nictitating membrane of *Elanus caeruleus* showing: (a) the two surfaces of nictitating membrane; the palpebral surface (ps), bulbar surface (bs) and the free margin (Fm) the leading edge (zigzag arrow), and fornix conjunctiva bulbi (F.c.b). and fornix conjunctiva palpebrae (F.c.p). (H& E, scale bar 20 µm). (b) the head of the membrane which is composed

of the free margin (Fm) and leading edge (arrowhead) and pigmented granules (black arrow) (H& E, scale bar 50 μm). (c) the structure of the palpebral surface (Ps) and bulbar surface (bs). the palpebral surface (Ps) of the nictitating membrane (Masson's trichromic, scale bar 50 μm). (d) the epithelium of the bulbar surface converts to columnar cells toward the fornix conjunctiva bulbi with numerous unicellular mucous glands (blue arrow) (Masson's trichromic scale bar 50 μm)

FIGURE 11. Photomicrograph of a semithin section nictitating membrane of*Elanus caeruleus* , showing (a) the layers of palpebral surface (Ps) with superficial cells have pale cytoplasm (zigzag arrow). (b) the bulbar surface (bs) with cytoplasmic extension (arrow) and goblet cell (arrowhead). (toluidine blue, scale bar 100 μm).

FIGURE 12. Transmission electron micrograph of the nictitating membrane of *Elanus caeruleus,* showing (a) flat superficial cells of the palpebral surface connected to each other by interdigitations of the cell membranes (zigzag arrow). (b) M cell in bulbar epithelium with short microvilli (arrowheads), enterocyte (I) and goblet cell (G). (c) vesicles (v) of various sizes in the epithelium of bulbar surface. (d) cytoplasmic extensions on the bulbar epithelium (Mic. Mag. X 3600).

FIGURE 13. Immunohistochemistry of different immune cells in the upper and lower eyelid of *Elanus caeruleus* (scale bar, 50 μm).

FIGURE 14. Immunohistochemistry of different immune cells in the nictitating membrane of *Elanus caeruleus* (scale bar, 50 μm).

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