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Descriptive histological analysis of the upper, lower, and third eyelids and the conjunctiva-associated lymphoid tissue in birds of prey

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Abstract

Backgroud In this study, we present data obtained using light microscopy for the histological analysis of the upper eyelid (*palpebra dorsalis*), lower eyelid (*palpebra ventralis*), and third eyelid (*palpebra tertia*) also known as the nictitating membrane. We characterized the organized conjunctiva-associated lymphoid tissue (CALT) in selected raptor species. The aim of this study is to compare the histological structures of these eyelids in owls and diurnal raptors to identify potential evolutionary links or independent adaptations to their environments.

Materials and methods We examined 34 individuals from 18 species representing Accipitriformes, Falconiformes, and Strigiformes, sourced from the Wrocław Zoological Garden (Poland), private bird collections (Poland), and wild birds found dead in the natural environment (Poland). The study involved morphometric analysis of the length and thickness of the tarsal plate of the lower eyelid. Microscopic evaluation included histological staining, using Masson-Goldner trichrome, Mayer's hematoxylin & eosin, Movat pentachrome (modified Russell-Movat), and picro-Mallory trichrome.

Results The structure of the eyelids in the analyzed bird orders proved to be highly diverse in terms of the presence of common features. The third eyelid, as well as CALT, exhibited the most variations morphological structures among the analyzed species. Strigiformes emerged as the most distinctive group of raptors, characterized by the greatest differences in eyelid morphology. This group of birds is not only distinct from other raptors but also internally diverse, with many significant differences observed between individual owl species.

Conclusion Despite some common features, the upper, lower, and third eyelids of raptors from the orders Accipitriformes, Falconiformes, and Strigiformes exhibit significant morphological variation. The third eyelid and conjunctiva-associated lymphoid tissue (CALT) display the most diverse structures among the analyzed species. Owls stand out as a group of raptors with the most distinct eyelid morphologies, both compared to other raptors and within their own group. The small number of birds may lead to difficulties in distinguishing individual variation from species-specific traits, as we cannot be certain whether the observed differences result from genetic or environmental factors specific to the individual birds or if they are traits typical for the species. To address this issue, further studies

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involving a larger number of individuals from the same species are necessary to more accurately determine whether the morphological traits described in this study are consistent within the species or if significant variation exists among individuals.

Keywords Accipitriformes, Birds, Conjunctiva-associated lymphoid tissue, Diffuse lymphocytes, Eyelids, Falconiformes, High endothelial venules, Histology, *Neornithes*, Strigiformes

Background

Birds have larger eyes relative to body weight than mammals [1–3], and raptor eyes are especially large compared with those of other birds [4], which indicates that vision is an important sensory modality for this avian group [1–3]. Many studies have focused on the anatomy of the eye and vision in owls [5, 6], Falconiformes, and Accipitriformes [3, 7]. However, the eyelids, which serve a very important protective function for such a large and valuable organ in birds, have not been thoroughly studied, and their histological characteristics are known only for a few species [8–20].

Functions of the eyelids in birds

According to *Nomina Anatomica Avium* [21], in birds we can distinguish the upper eyelid (*palpebra dorsalis*), lower eyelid (*palpebra ventralis*) and third eyelid (*palpebra tertia*), also known as the nictitating membrane. The upper eyelid in most diurnal birds is short and thick, whereas the lower eyelid is longer, thinner, and more mobile. However, in nocturnal species, the upper eyelid is larger and more mobile [11, 21–23]. Closure of the eye takes place through the movement of the lower eyelid, which completely covers the cornea and contains a fibrous tarsal plate (in birds of prey, it may be a cartilaginous tarsal plate) [11, 14, 21–23]. The lower eyelid margin in birds is thickened and has a variable number of marginal folds, enabling the stretching of this eyelid during the closure of the eye [14, 21, 22]. Contrary to mammals, the third eyelid in birds is elastic and characterized by the presence of two muscles: the quadratus third eyelid muscle and the pyramidal third eyelid muscle, which are responsible for the so-called active movement of the third eyelid [14, 21, 22]. Moreover, the free margin of the third eyelid has thick folds, which distribute fluids and detritus on the corneal anterior epithelium [12, 13, 21].

The upper, lower and third eyelids in birds protect the eyeball against mechanical injuries and foreign bodies, clear debris from the external surface, and regulate the flow of light into the eye [12, 24]. Their function is also to spread precorneal tear film on the corneal surface and prevent desiccation of the precorneal tear film during the bird's flight [11, 26]. Moreover, birds have well-developed conjunctiva-associated lymphoid tissue (CALT) and Harderian glands, which provide nutrients and antibodies to maintain the health of the cornea and conjunctival sacs, and also moisturize the eye [11, 12, 14, 25, 26]. According to van Ginkel et al. [27], CALT and the Harderian gland can form one main eye-associated lymphoid structure. The CALT in birds is composed of lymphoid follicles (termed the follicle-associated epithelium FAE) located directly under the lower conjunctival epithelium and ocular epithelium of the third eyelid, and it diffuses lymphocytes found in the subepithelial lamina propria [12, 28]. The lymphocytes from the bloodstream enter the lymphoid tissue principally via specialized high endothelial venules (HEV) [27], and in birds (e.g. Bilgorey Goose *Anser anser domestica*), these lymphocytes are located both within and around the HEV [13]. Fix and Arp [29] and van Ginkel et al. [27] reported that in birds, CALT contains cytokine-producing T-cells, B-cells, and IgA-secreting plasma cells, which are important in maintaining ocular immunity, particularly important for birds.

Definition of the raptor/bird of prey

Many researchers have argued about the definition of a raptor and a bird of prey [30–36]. Some authors use the terms raptor and bird of prey as synonyms [32, 33, 35, 36], while other researchers believe that the term "bird of prey" denotes every bird that preys on other living animals [30, 31], and the attribute "raptorial" can refer only to bird species that feed specifically on vertebrates [34]. Therefore, to date, depending on morphological, ecological, and taxonomic criteria, various groups of birds are considered birds of prey. Traditionally, this group included birds with distinctive morphological features, such as keen eyes (for detecting prey), hooked bills (for killing and consuming prey), and long, sharp talons that can seize or grip [30, 37, 38]. However, this definition is imprecise because some of these morphological features can also be found in members of bird orders that are not considered raptors, such as parrots (Order: Psittaciformes) [36]. On the other hand, an ecological trait largely accepted as a characteristic of raptorial birds is hunting live vertebrates and eating their meat [31, 37, 39]. However, this behavior can also be found in members of bird orders that are not considered raptors, such as shrikes and ravens (Order: Passeriformes) [40–42], and some seabirds (Order: Charadriiformes) [37, 43–45]. Furthermore, vultures are often considered raptors despite most of the species being scavengers [46], with some species exclusively (Indian Vulture *Gyps indicus*) or almost exclusively (Himalayan Griffon *Gyps himalayensis*) feed on carrion [47, 48], prefer to eat bone marrow (Bearded

Vulture *Gypaetus barbatus*), or even, like the Palm-nut Vulture (*Gypohierax angolensis*), are frugivorous [49–51]. Moreover, many large raptors such as Bateleur (*Terathopius ecaudatus*) and fish eagles (*Haliaeetus* spp.), are also scavengers [50, 51]. Therefore, a raptorial lifestyle alone cannot be an exclusive or precise criterion for defining birds of prey [36, 38]. The definition proposed by McClure et al. [36] considers as raptors, or birds of prey, all species within orders that have evolved from a raptorial landbird lineage and in which most species have maintained their raptorial lifestyle as derived from their common ancestor. This definition combines phylogeny, morphological, and ecological features, and highlights the importance of using evolutionary history to describe patterns of shared common ancestry. In light of this definition, the following orders are considered raptors/birds of prey in this research: Accipitriformes, Cathartiformes, Strigiformes, Cariamiformes, and Falconiformes [34, 36, 52, 53]. This approach allows us to avoid the ambiguity that often is a results of considering only morphological traits or only behavioral traits for identifying raptors, as it was the case with owls (Order: Strigiformes) [31, 38, 50, 54].

Because so far research on the organ of vision in birds of prey has focused on the eyeball itself [3, 5–7] it is interesting to compare the structures of the eyelids, and especially the nictitating membrane, between owl species with different daily activities and between typical diurnal raptors representing the orders Accipitriformes and Falconiformes, which is the main aim of this study. Research conducted allows us to determine whether any structural features of the eyelids can indicate the origin from a common raptorial landbird ancestor of these groups or whether they are only the result of an independent adaptation to the environment.

Materials and methods

Collection of specimens

This study was conducted on 18 species of birds representing 4 families: Accipitridae, Falconidae, Strigidae and Tytonidae [55]. Research material comes from: Wrocław Zoological Garden (Poland), private bird collections (Poland) and individuals of wild birds found dead in natural environment (Poland). The exact location where each individual was collected is listed in Table 1. Tissue material was collected from 2019 to 2023. The number of examined individuals and their species affiliation are shown in Table 1. The examined birds were not killed for the purpose of this study and all died under natural circumstances.

Microscopic study

Entire undamaged eyelids (upper eyelid, lower eyelid and third eyelid) of all birds were inspected visually using a Zeiss Stemi 2000-C stereoscopic microscope (Carl Zeiss, Jena, Germany). The terminology used follows the prevailing veterinary nomenclature – Nomina Anatomica Avium [21], and Nomina Histologica Veterinaria [56]. The length and thickness of the tarsal plate of the lower eyelid were conducted bilaterally using a GRIP digital caliper with a resolution of 0.01 mm and an accuracy of +/- 0.02 mm (> 100 mm) (Handy Worth, Poland).

Tissue processing for light microscopy

The upper, lower and third eyelids were collected from each individual for testing. Samples were placed in 4% buffered formaldehyde for at least 72 h and then rinsed in running water for 24 h. The samples were dehydrated using 75%, 96%, and 100% ethanol solution processed in a vacuum tissue processor – ETP (RVG3, Intelsint, Italy) and embedded in paraffin. The samples were sectioned using a Micron HM310 microtome into 5 µm sections. The following histological staining techniques were performed: Masson-Goldner trichrome, Mayer's hematoxylin & eosin, Movat pentachrome (modified Russell-Movat), and picro-Mallory trichrome [57, 58]. Histological staining was performed at the Division of Histology, Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences. Histological preparations were observed using a Zeiss Axio Scope A1 light microscope (Carl Zeiss, Jena, Germany).

For the Steppe Eagle (*Aquila nipalensis*), White-tailed Eagle (*Haliaeetus albicilla*), Little Owl (*Athene noctua*) and Tawny Owl (*Strix aluco*), we used previously prepared histological samples [14] that were subjected to additional analysis.

Results

Histologically, in all examined birds, the upper eyelids and lower eyelids were composed of an external surface (anterior palpebral surface) and an internal surface (posterior palpebral surface). The posterior palpebral surface of the upper eyelid consisted of the marginal zone and the bulbar zone, while the posterior palpebral surface of the lower eyelid had the marginal zone, the plate zone and the bulbar zone. The third eyelid is composed of the marginal plait, leading edge, palpebral surface with palpebral folds and bulbar surface with bulbar folds (Fig. 1).

Common eyelid features across Strigiformes, Accipitriformes, and Falconiformes

A common feature among all examined bird species is the keratinized stratified squamous epithelium covering

Table 1 Taxonomical affiliation of the examined birds according to Clements et al., 2023. The table contains the number of examined individuals, the source of biological material and information on hunting activity of analyzed species. ([1]Mindell et al., 2018; [2]Mikkola, 1983; [3]del Hoyo et al., 1999; [4]Holt et al., 2020a; [5]Duncan and Duncan, 2020)

Order	Family	Species		Activity	No. of individuals	Source
Accipitriformes	Accipitridae	Eurasian Goshawk	*Accipiter gentilis*	diurnal[1]	2	captive (Poland)
Accipitriformes	Accipitridae	Eurasian Sparrowhawk	*Accipiter nisus*	diurnal[1]	3	1 individual found in outskirts of a horse stud farm (Kątna, Poland); 1 individual found in a large city park (Wrocław, Poland); 1 individual found in the outskirts of woods near a village (Chrząstawa Wielka, Poland)
Accipitriformes	Accipitridae	Steppe Eagle	*Aquila nipalensis*	diurnal[1]	1	histologic specimen (Klećkowska et al., 2017)
Accipitriformes	Accipitridae	Common Buzzard	*Buteo buteo*	diurnal[1]	4	1 individual from Wild Animal Rehabilitation Center (Kątna, Poland); 1 individual found near the expressway on the outskirts of town (Siechnice, Poland); 1 individual found in a meadow near a village (Chwałowice, Poland); 1 individual found in the outskirts of woods near a village (Chrząstawa Wielka, Poland)
Accipitriformes	Accipitridae	Hen Harrier	*Circus cyaneus*	diurnal[1]	1	found on the embankments over the Oder river (Wrocław, Poland)
Accipitriformes	Accipitridae	White-tailed Eagle	*Haliaeetus albicilla*	diurnal[1]	1	histologic specimen (Klećkowska-Nawrot et al., 2017)
Accipitriformes	Accipitidae	European Honey-buzzard	*Pernis apivorus*	diurnal[1]	2	captive (Poland)
Falconiformes	Falconidae	Peregrine Falcon	*Falco peregrinus*	diurnal[1,]	2	captive (Poland)
Falconiformes	Falconidae	Eurasian Kestrel	*Falco tinnunculus*	diurnal[1]	5	3 individuals found near a multi-story residential building (Wrocław, Poland); 1 individual from Wild Animal Rehabilitation Center (Kątna, Poland); 1 individual found in rural areas of town (Siechnice, Poland)
Strigiformes	Strigidae	Boreal Owl	*Aegolius funereus*	diurnal and nocturnal[2]	1	Wrocław Zoological Garden (Poland)
Strigiformes	Strigidae	Little Owl	*Athene noctua*	nocturnal, crepuscular and diurnal[4]	1	histologic specimen (Klećkowska-Nawrot et al., 2017)
Strigiformes	Strigidae	Eurasian Eagle-Owl	*Bubo bubo*	nocturnal, crepuscular and diurnal[4]	1	Wrocław Zoological Garden (Poland)
Strigiformes	Strigidae	Snowy Owl	*Bubo scandiacus*	diurnal and nocturnal[2]	1	Wrocław Zoological Garden (Poland)
Strigiformes	Strigidae	Philippine Scops-Owl	*Otus megalotis*	nocturnal[3]	1	Wrocław Zoological Garden (Poland)
Strigiformes	Strigidae	Tawny Owl	*Strix aluco*	nocturnal[2]	2	histologic specimen (Klećkowska-Nawrot et al., 2017)
Strigiformes	Strigidae	Ural Owl	*Strix uralensis*	nocturnal and crepuscular[2]	2	Wrocław Zoological Garden (Poland)
Strigiformes	Strigidae	Northern Hawk Owl	*Surnia ulula*	diurnal and nocturnal[5]	2	Wrocław Zoological Garden (Poland)
Strigiformes	Tytonidae	Western Barn Owl	*Tyto alba*	nocturnal and crepuscular[2]	1	Wrocław Zoological Garden (Poland)

the anterior surface of the upper eyelid and the lower eyelid (Fig. 2a and c). A densely woven lamina propria, consisting of collagen fibers, delicately marked elastic and reticular fibers, histiocytes, and fibrocytes, was observed directly beneath the anterior surface of the eyelid (Fig. 2a and c). Skin folds and folds of conjunctiva were present in both eyelids (Fig. 2d, f, g and i). Sebaceous and sweat glands were located at the base of the "hair feathers" alongside smooth muscle of feathers and elastic tendon (Fig. 2j and l). The marginal zone in the eyelids

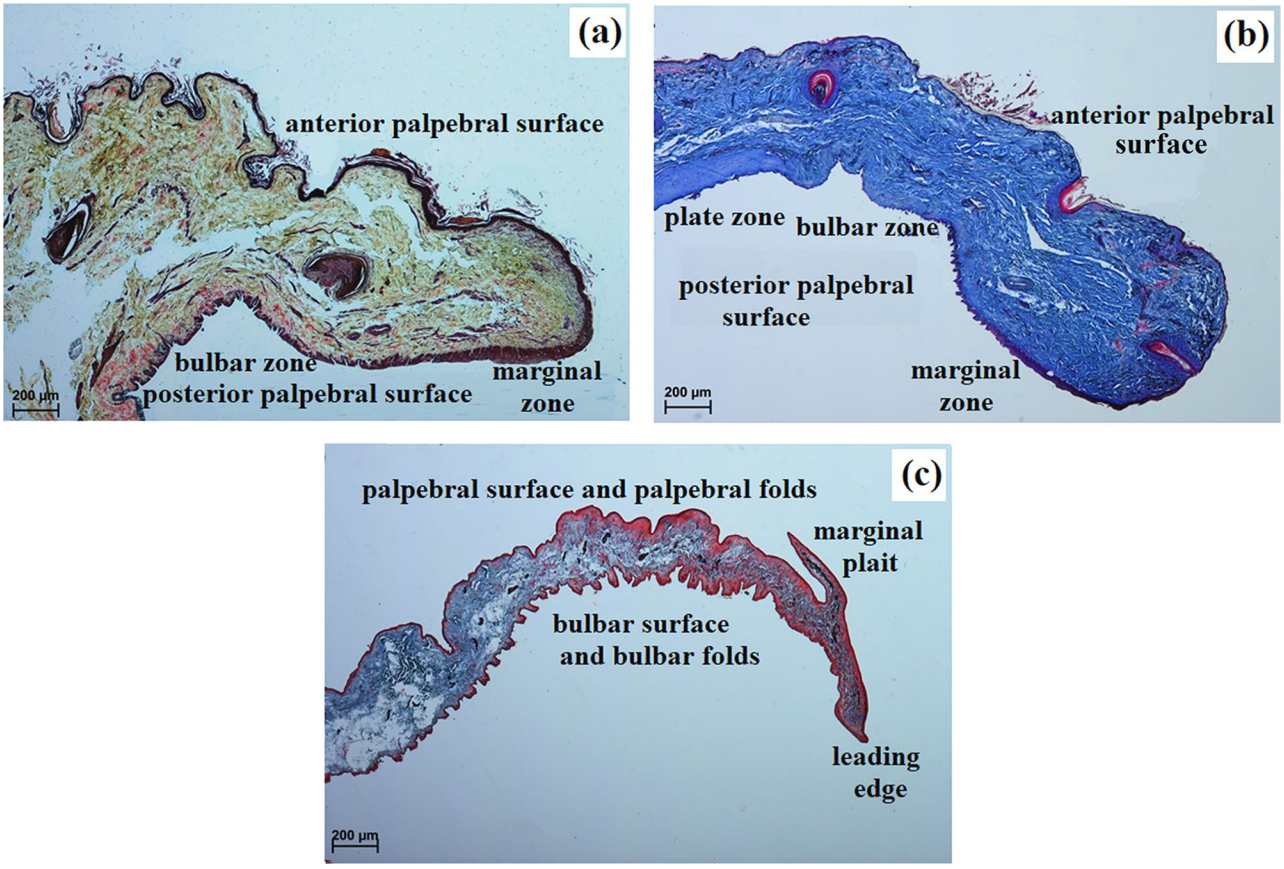

Fig. 1 The overall structure of the upper eyelid (**a**), lower eyelid (**b**) and third eyelid (**c**) in the examined birds. (**a**) – Eurasian Eagle-Owl; (**b**) – Peregrine Falcon; (**c**) – Eurasian Sparrowhawk. **a** – Movat pentachrome (modified Russell-Movat) stain; **b**, **c** – picro-Mallory trichrome stain. Scale bars: **a** – **b** = 200 µm

was covered by stratified columnar epithelium (Fig. 2m and o). The stroma of eyelids was consisted of compact fibrous connective tissue with irregular weaving including collagen fibers, elastic fibers, reticular fibers, bundles of muscle, nerves and blood vessels (Fig. 2p and s). The posterior surface in the upper and lower eyelids was covered with non-keratinized stratified squamous epithelium, containing a relatively modest number of well-differentiated goblet cells (Fig. 2t and w). The tarsal plate in the lower eyelid was oval and composed of compact fibrous connective tissue with irregular weaving (collagen fibers, elastic fibers and reticular fibers, fibrocytes), with blood vessels either present individually or surrounded by melanocytes (Fig. 2x and z).

Features common to all three bird orders were also identified in the third eyelid. The marginal plait and leading edge were lined by non-keratinized stratified squamous epithelium (Fig. 3a and b). The palpebral surface was lined with non-keratinized stratified squamous epithelium containing numerous goblet cells, while the bulbar surface was lined with non-keratinized stratified columnar epithelium (Fig. 3d and i). The stoma of the third eyelid was composed of compact fibrous tissue with regular weaving (Fig. 3j and l). The palpebral surface formed thick folds, while the bulbar surface of the leading edges formed thin folds (Fig. 3m and o).

The histological analysis of the upper and lower eyelids showed that the lamina propria in Accipitriformes, Falconiformes and Strigiformes was composed of compact fibrous tissue with irregular weaving. Similarly, well-developed muscle fibers were observed in the stroma of both eyelids in representatives of all analyzed orders, except for the Steppe Eagle and the White-tailed Eagle (Fig. 4m and o).

Distinctive features of Strigiformes compared to Accipitriformes and Falconiformes

Histological analysis of the upper and lower eyelids in all analyzed owl species showed numerous adipose cells and clusters of melanocytes in the lamina propria (Fig. 4a and c). A distinctive feature that separates Strigiformes from the other two orders is the presence of a large adipose pad in both eyelids (Fig. 4d and k). Moreover, the marginal plait and the leading edge of the third eyelid in the examined owl species were very thick, unlike those of the other two bird orders (Fig. 5a and i). Large differences were also observed between the three analyzed bird orders in the number of palpebral and conjunctival folds.

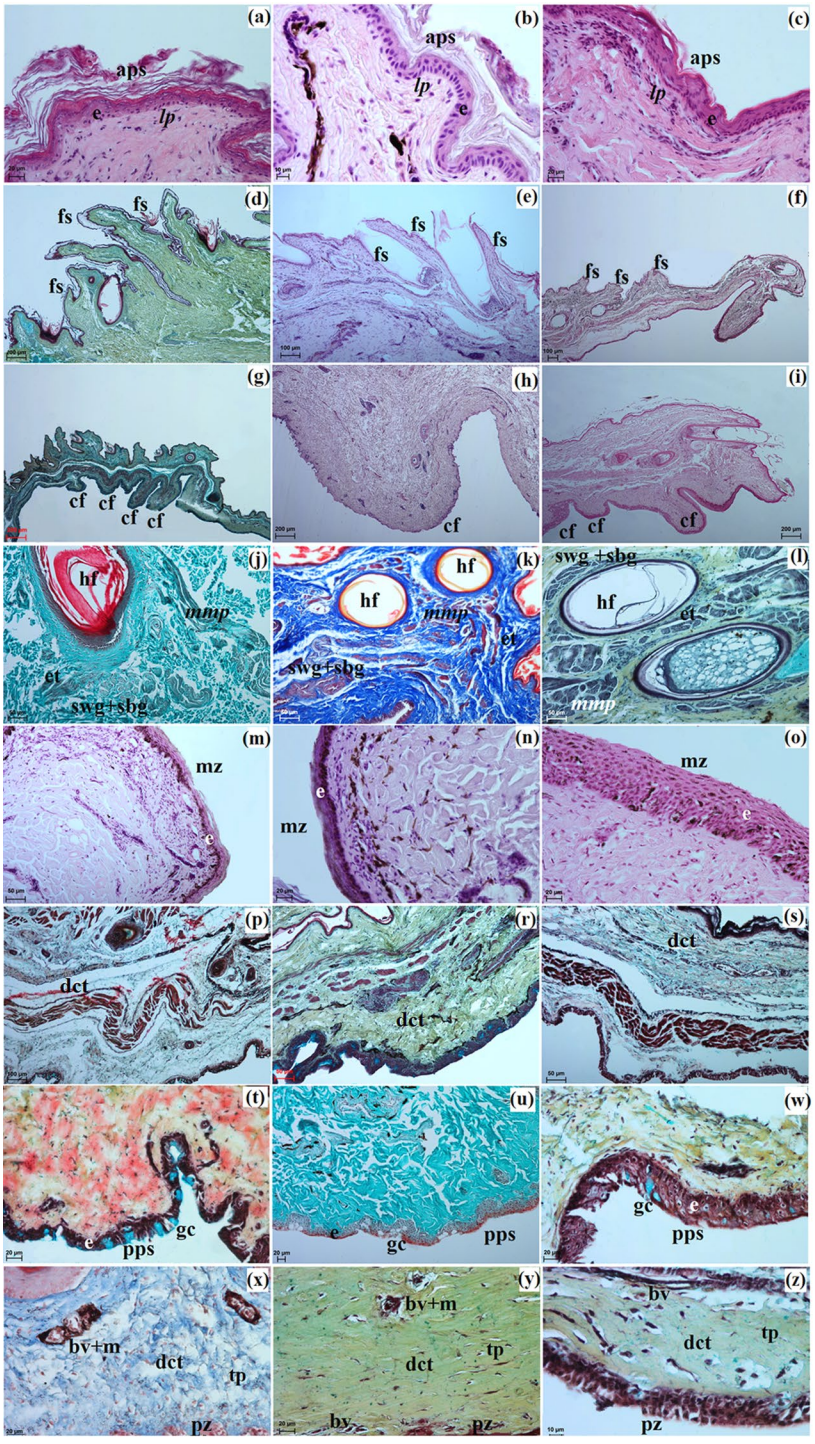

Fig. 2 Common structural features of the upper eyelid and lower eyelid on the Strigiformes (**a**, **d**, **g**, **j**, **m**, **p**, **t**, **x**), Accipitriformes (**b**, **e**, **h**, **k**, **n**, **r**, **u**, **y**), and Falconiformes (**c**, **f**, **i**, **l**, **o**, **s**, **w**, **z**). (**a**) – Western Barn Owl; (**b**) and (**k**) – Eurasian Sparrowhawk; (**c**), (**f**), (**s**) and (**w**) – Peregrine Falcon; (**d**) – Ural owl; (**e**) – Eurasian Goshawk; (**g**) – Boreal Owl; (**h**) – Steppe Eagle; (**i**), (**l**), (**o**) and (**z**) – Eurasian Kestrel; (**j**) – Philippine Scops-Owl; (**m**) – Eurasian Eagle-Owl; (**n**) and (**y**) – European Honey-buzzard; (**r**) – Hen Harrier; (**p**) – Little Owl; (**u**) – White-tailed Eagle; (**t**) – Northern Hawk Owl; (**x**) – Tawny Owl. aps – anterior palpebral surface, bv – blood vessels, bv + m – blood vessels and melanocytes, cf – conjunctival folds, dct – dense connective tissue, e – epithelium, et – elastic tendon, fs – folds of skin, gc – goblet cells, hf – „hair feathers", *lp* – *lamina propria*, *mmp* – *mm. pennarum*, mz – marginal zone, pps – posterior palpebral surface, pz – plate zone, swg + sbg – sweat glands and sebaceous glands, tp – tarsal plate. **a** – **c**, **e**, **f**, **h**, **i**, **m** – o = Mayer's H&E stain; j, u = Masson Goldner trichrome stain; **d**, **g**, **l**, **p** – **t**, **w**, **y**, **z** = Movat pentachrome (modified Russell-Movat) stain; k, x = picro-Mallory trichrome stain. Scale bars: **d**, **g**, **h**, **i** = 200 µm; **e**, **f**, *p* = 100 µm; **j**, **k**, **l**, **m**, **r**, **s** = 50 µm; **a**, **c**, **n**, **o**, **t**, **u**, **w**, **x**, **y** = 20 µm; **b**, **z** = 10 µm

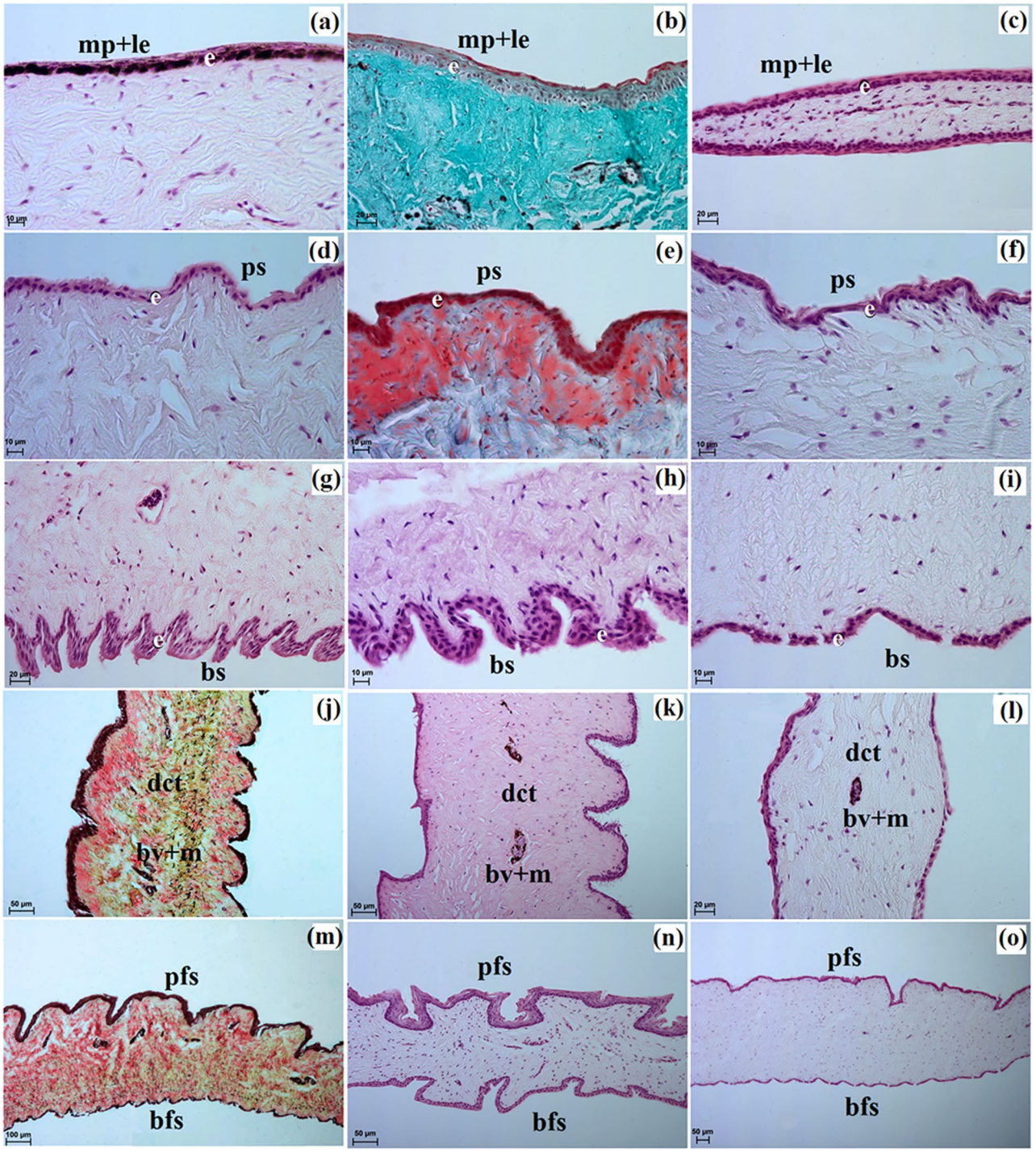

Fig. 3 Third eyelid structural similarities in Strigiformes (**a**, **d**, **g**, **j**, **m**), Accipitriformes (**b**, **e**, **h**, **k**, **n**), and Falconiformes (**c**, **f**, **i**, **l**, **o**). (**a**) – Eurasian Eagle-Owl; (**b**) – White-tailed Eagle; (**c**), (**i**) and (**o**) – Peregrine Falcon; (**d**) – Snowy Owl; (**e**) – Eurasian Sparrowhawk; (**f**) and (**l**) – Eurasian Kestrel; (**g**) – Boreal Owl; (**h**) – Hen Harrier; (**k**) – Eurasian Goshawk; (**j**) and (**m**) – Northern Hawk Owl; (**n**) – Steppe Eagle. bfs – bulbar folds, bs – bulbar surface, bv + m – blood vessels and melanocytes, **e** – epithelium, dct – dense connective tissue, mp + le–marginal plate and leading edge, pfs – palpebral folds, ps – palpebral surface. **a**, **c**, **f** – **i**, **k**, **l**, **n**, **o** = Mayer's H&E stain; b = Masson Goldner trichrome stain; **d**, **j**, m = Movat pentachrome (modified Russell-Movat) stain; **e** = picro-Mallory trichrome stain. Scale bars: m = 100 µm; **j**, **k**, **n**, o = 50 µm; b, **c**, **g**, l = 20 µm; **a**, **d**, **e**, **f**, **h**, i = 10 µm

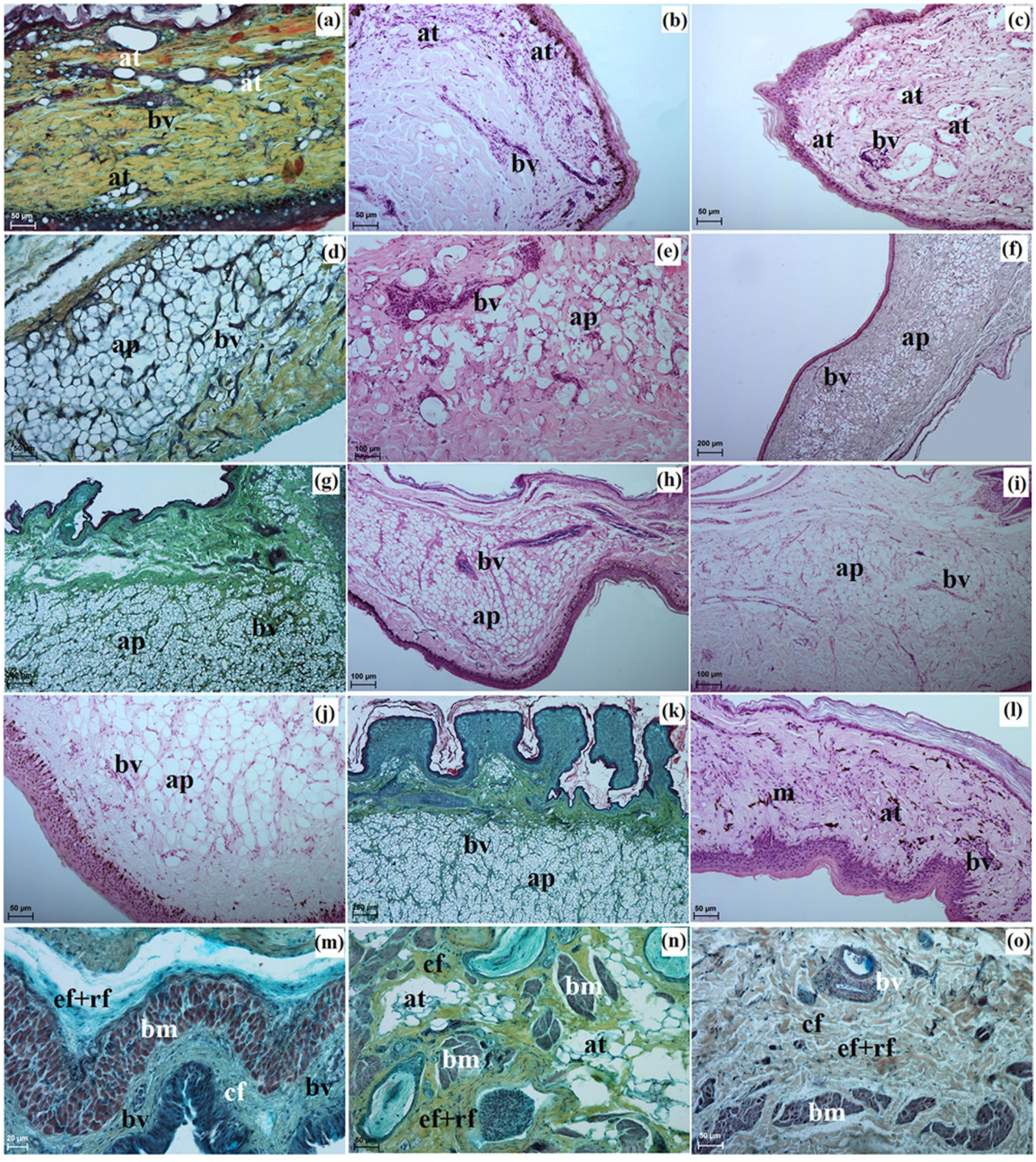

Fig. 4 Distinctive structural features of the upper and lower eyelids in Strigiformes compared to Accipitriformes and Falconiformes. (**a**), (**e**) and (**n**) – Northern Hawk Owl; (**b**), (**f**) and (**o**) – Eurasian Eagle-Owl; (**c**) and (**i**) – Snowy Owl; (**d**) and (**m**) – Boreal Owl; (**g**) – Ural Owl; (**h**) and (**o**) – Tawny Owl; (**l**) – Little Owl; (**j**) – Philippine Scops-Owl; (**k**) – Western Barn Owl. at – adipose tissue, ap – adipose pad, bm – bundle of muscle, bv – blood vessels, cf. – collagen fibres, ef + rf – elastic fibres and reticular fibres. **b**, **c**, **e**, **f**, **h**, **i**, **j**, l = Mayer's H&E stain; **a**, **d**, **g**, **k**, **m** – o = Movat pentachrome (modified Russel-Movat) stain. Scale bars: **f**, **g**, **k** = 200 µ**m**; **e**, **h**, i = 100 µm; **a** – **d**, **l**, **j**, **n**, **o** = 50 µm; m = 20 µm

In Strigiformes, these ranged from 10 to 34 and 29 to 70, respectively, compared to 6 to 30 and 29 to 45 in Accipitriformes and 10 to 23 and 23 to 33 in Falconiformes species. Consequently, the third eyelid of Strigiformes was longer (Fig. 5a and i; Table 3).

Distinctive features among Owl species

The first feature, observed in the stroma of the third eyelid in the Northern Hawk Owl (*Surnia ulula*) and Western Barn Owl (*Tyto alba*), was the presence of clusters of melanocytes arranged around blood vessels, which differed from other owl species (Fig. 5j and s).

Another feature was the varying number of layers of the cells forming the anterior epithelium in the upper and lower eyelids from 2 to 3 to 4–5 in Boreal Owl (*Aegolius funereus*), Eurasian Eagle-Owl (*Bubo bubo*), Ural Owl (*Strix uralensis*), Little Owl, Tawny Owl, and Philippine Scops-Owl (*Otus megalotis*), while ranging from 6 to 7 to 15 layers in the Snowy Owl (*Bubo scandiacus*), Northern Hawk Owl and Western Barn Owl (Fig. 6a and i; Table 2). The wide and short skin folds present in both eyelids among the studied owls were most commonly found in the Boreal Owl, Little Owl, Eurasian Eagle-Owl, Philippine Scops-Owl, Tawny Owl, and Ural Owl (Fig. 6j and o; Table 2). In the Northern Hawk Owl and Snowy Owl, the skin folds were also wide but much longer (Fig. 6p and n; Table 2). In contrast, in the case of Western Barn Owl, the skin folds were very wide and short, resembling bricks placed next to each other (Fig. 6s; Table 2). Only in the lamina propria beneath the anterior palpebral epithelium of both eyelids of Northern Hawk Owl clusters of melanin were observed (Fig. 6g; Table 2).

In the case of the marginal zone epithelium, large differences between owl species have also been observed: from 7 to 9–11 layers in the Boreal Owl, Little Owl, Eurasian Eagle-Owl, Snowy Owl, and from 10 to 23 layers in the Philippine Scops-Owl, Tawny Owl, Ural Owl, Northern Hawk Owl, and Western Barn Owl (Fig. 7a and i; Table 2). An important difference observed between Strigiformes species is the absence of an adipose pad in the upper and lower eyelids of the Little Owl (Fig. 4l). Differences between the analyzed owl species were also noted in the length and width of the tarsal plate on the lower eyelids. A large tarsal plate was observed in the Eurasian Eagle-Owl and Ural Owl, whereas a small tarsal plate was present in the Boreal Owl, Little Owl, and Philippine Scops-Owl (Table 2). Moreover, in the lamina propria of the marginal zone, large accumulations of melanocyte clusters were present in the Boreal Owl, Little Owl, and Eurasian Eagle-Owl (Fig. 7a, d and e; Table 2). In this marginal zone, numerous blood vessels were present in Tawny Owl (Fig. 7g; Table 2), and numerous adipose cells were found in Snowy Owl and Northern Hawk Owl (Fig. 7c and e; Table 2). Melanin granules were observed in the epithelium of the marginal zone of both eyelids in most of the studied species, except for the Tawny Owl and Ural Owl (Fig. 7f and g). Diversity was also demonstrated in the number of layers of nucleated cells in the posterior epithelium, from 3 to 4 to 16–18 layers in the upper eyelid and from 4 to 5 to 20–22 layers in the lower eyelids (Fig. 8a and i; Table 2).

In the lower eyelid of Eurasian Eagle-Owl, the presence of conjunctiva-associated lymphoid tissue (CALT) was identified, whereas in the remaining owl species lymphoid follicles or diffuse lymphocytes with high endothelial venules (HEV) were observed (Fig. 8j and w; Table 2). Significant differences among the analyzed Strigiformes species were noted in the number of cell layers forming the epithelium in the marginal plait with the leading edge of the third eyelid (ranging from 3 to 10), the palpebral surface of the third eyelid (ranging from 2 to 11), and the bulbar surface of the third eyelid (ranging from 3 to 13) (Fig. 9a and s; Table 3).

Distinctive features of the eyelids in Accipitriformes and Falconiformes

A common feature among the analyzed Accipitriformes and Falconiformes species was the similar number of layers of nucleated cells forming the anterior surface of the upper and lower eyelids (Table 2). This ranged from 2 to 3–4 layers in the Eurasian Goshawk (*Accipiter gentilis*), Hen Harrier (*Circus cyaneus*), and European Honey-buzzard (*Pernis apivorus*) (Fig. 10a and c). In contrast, the Eurasian Sparrowhawk (*Accipiter nisus*), Common Buzzard (*Buteo buteo*), and Eurasian Kestrel (*Falco tinnunculus*) had 3–4 to 5 layers of cells (Fig. 10d and f). The number of nucleated cell layers varied between 6 and 8 in Steppe Eagle and Peregrine Falcon (*Falco peregrinus*), while the White-tailed Eagle had the highest number layers, ranging from 9 to 10 (Fig. 10g and i; Table 2).

The presence of melanin clusters in the lamina propria beneath the anterior epithelium of the upper and lower eyelids was a common feature of the Eurasian Sparrowhawk, Common Buzzard, Hen Harrier, European Honey-buzzard, and Peregrine Falcon (Fig. 10b, e and h 10j 10l, 10n, 10s; Table 2). In contrast, the absence of melanin clusters in lamina propria was a shared characteristic of the Eurasian Goshawk, Steppe Eagle, White-tailed Eagle, and Eurasian Kestrel (Fig. 10a, f, g, i, m, o and r; Table 2). The distribution of melanocytes in the epithelium of the marginal zone of upper and lower eyelids was also noteworthy. In the Eurasian Goshawk, Common Buzzard, Steppe Eagle, and Peregrine Falcon, melanocytes were sparse and were primarily located in the basal lamina epithelium (Fig. 11a and d; Table 2). However, in the Eurasian Sparrowhawk, Hen Harrier, White-tailed Eagle, European Honey-buzzard, and Eurasian Kestrel, melanocytes were abundant and distributed throughout

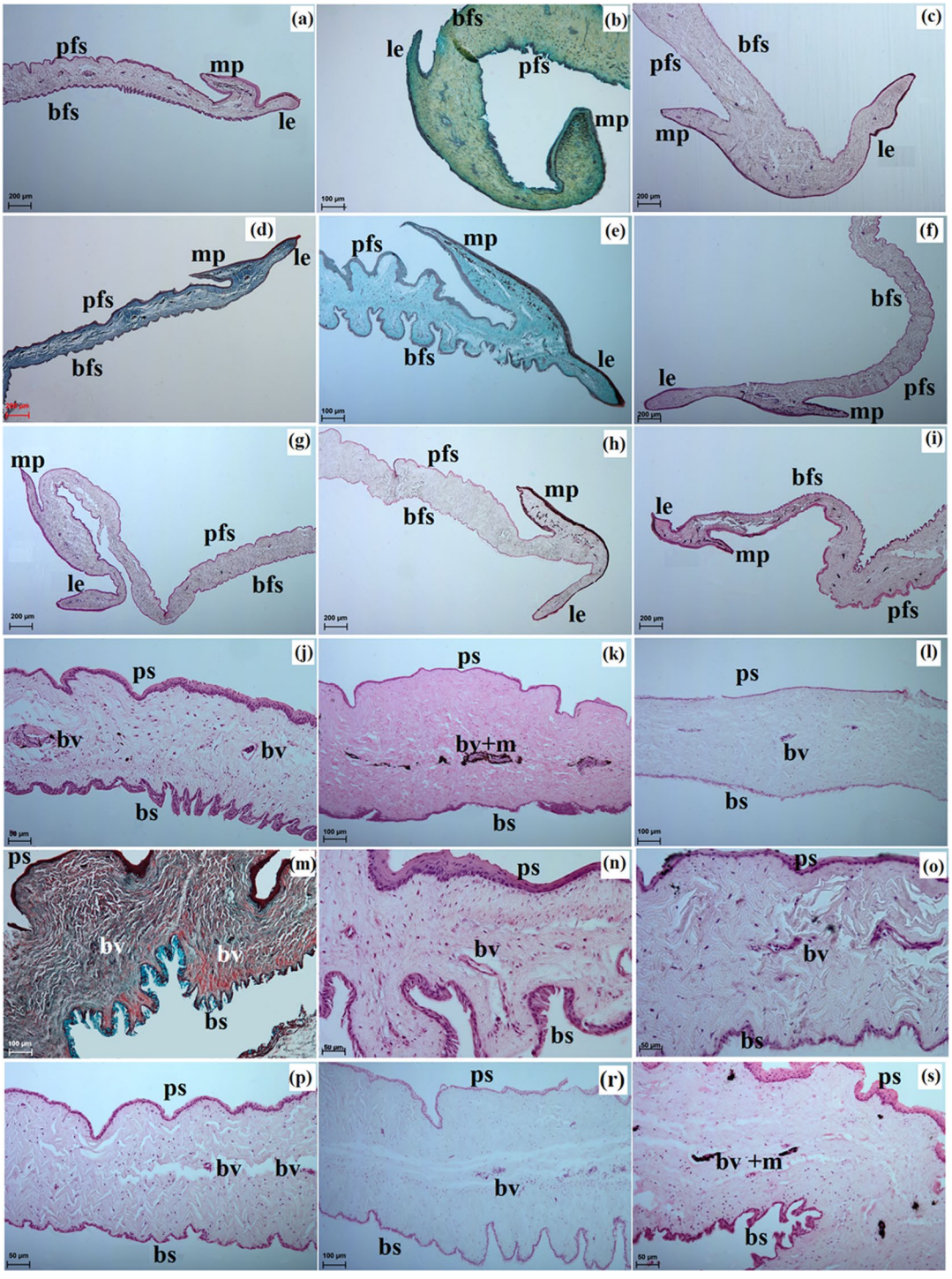

Fig. 5 Distinctive structural features of the third eyelid in Strigiformes species. (**a**) and (**j**) – Boreal Owl; (**b**) and (**k**) – Northern Hawk Owl; (**c**) and (**l**) – Eurasian Eagle-Owl; (d) and (m) – Ural Owl; (**e**) and (**n**) – Little Owl; (**f**) and (**o**) – Tawny Owl; (**g**) and (**p**) – Snowy Owl; (**h**) and (**r**) – Philippine Scops-Owl; (**i**) and (**s**) – Western Barn Owl. bfs – bulbar folds, bv – blood vessels, bv + m – blood vessels and melanocytes, le – leading edge, mp – marginal plait, mz – marginal zone, pfs – palpebral folds. a, c, f – l, n – s = Mayer's H&E stain; b, e, m = Movat pentachrome (modified Russell-Movat) stain; d = picro-Mallory trichrome stain. Scale bars: **a**, **c**, **d**, **f**, **g** – i = 200 µm; **b**, **e**, **k**, **l**, **m**, *r* = 100 µm; **j**, **n**, **o**, **p**, s = 50 µm

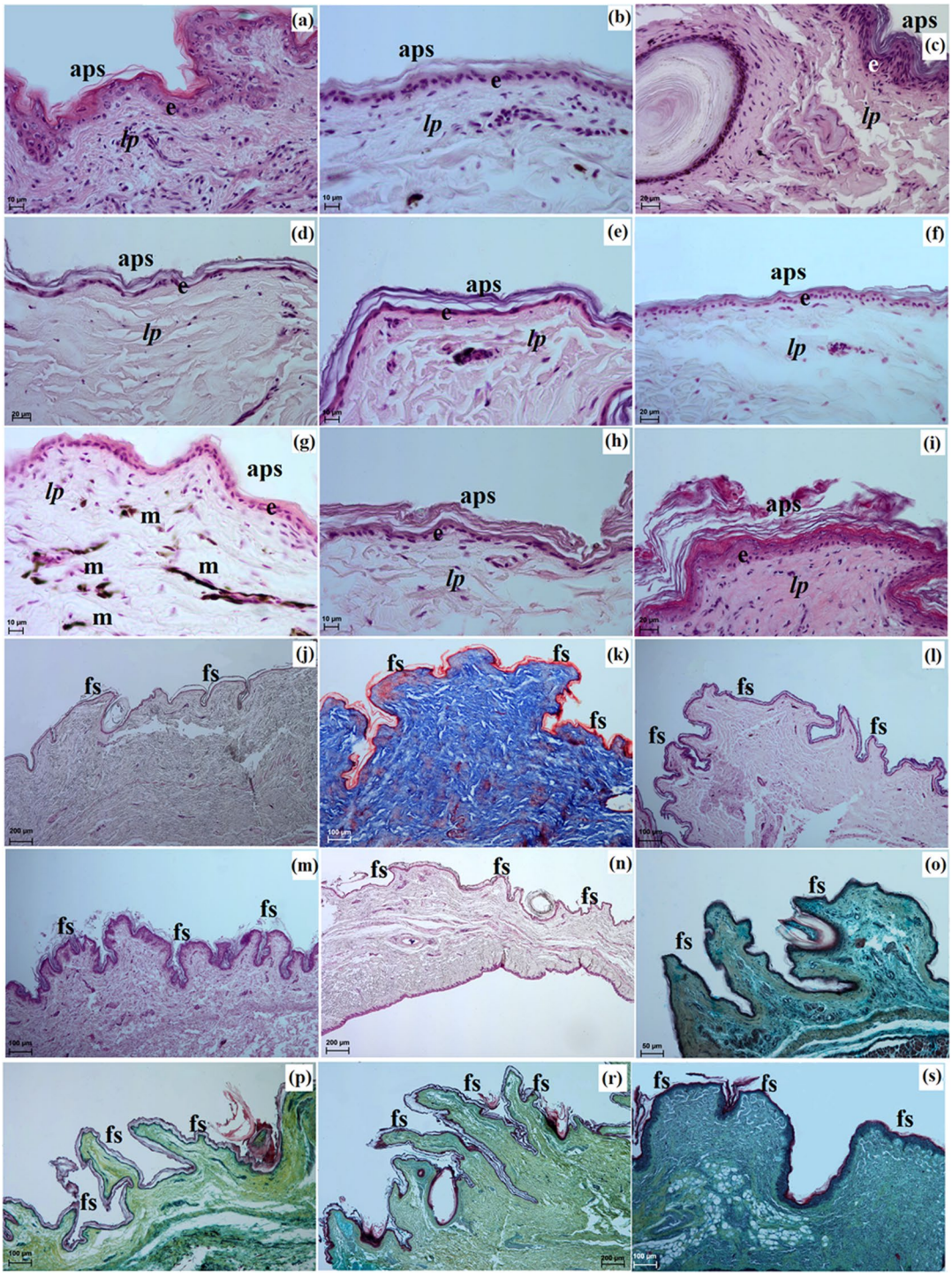

Fig. 6 Structural differences in the upper and lower eyelids across Strigiformes. (**a**) and (**o**) – Boreal Owl; (**b**) and (j) – Eurasian Eagle-Owl; (**c**) and (**r**) – Ural Owl; (d) and (k) – Little Owl; (**g**) and (**l**) – Tawny Owl; (**h**) and (**m**) – Philippine Scops-Owl; (**g**) and (**p**) – Northern Hawk Owl; (**h**) and (**n**) – Snowy Owl; (**i**) and (**s**) – Western Barn Owl. aps – anterior palpebral surface, e – epithelium, fs – folds of skin, *lp* – *lamina propria*.**a** – **j**, **j**, **l** – n = Mayer's H&E stain; o – s = Movat pentachrome (modified Russell-Movat) stain; k = picro-Mallory trichrome stain. Scale bars: **j**, **n**, *r* = 200 µm; **k** – **m**, **p**, s = 100 µm; o = 50 µm; **c**, **d**, **f**, i = 20 µm; **a**, **b**, **e**, **g**, h = 10 µm

Table 2 Characteristic features of the structure of upper eyelid and lower eyelid in the examined birds. CALT – conjunctiva-associated lymphoid tissue, LF – lymphoid follicle, DL – diffuse lymphocytes, HEV – high endothelial venules, (+) – poorly marked melanocytes in the epithelium, (+++) – numerous accumulations of melanocytes in the epithelium, (+) – presence, (-) – absence

Order	Species	Epithelium of the anterior palpebral surface	Melanocytes in Lamina propria	Folds of the skin	Epithelium of the marginal zone	Localization of melanocytes of the marginal zone epithelium	Presence /absence of the melanocytes in the marginal zone stroma	Length of the tarsal plate [mm]	Width of the tarsal plate [mm]	Epithelium of the posterior surface on the upper eyelid	Epithelium of the posterior surface on the lower eyelid	CALT	LF	DL	HEV
Accipitriformes	*Accipiter gentilis*	2–3–4	-	long and narrow	17–20	+ (basal lamina)	-	8–9	10–11	5–8	3–5	+	-	-	-
Accipitriformes	*Accipiter nisus*	3–4–5	+	short and wide	10–12	+++ (all epithelium)	+	11–12	6–7	4–5	4–5	+	-	-	-
Accipitriformes	*Aquila nipalensis*	6–8	-	short and wide	16–19	+ (basal lamina)	+	15–16	10–11	6–7	10–11	+	-	-	-
Accipitriformes	*Buteo buteo*	3–4–5	+	long and narrow	15–16	+ (basal lamina)	+	12–13	10–11	3–5	7–9	+	-	-	-
Accipitriformes	*Circus cyaneus*	2–3–4	+	long and narrow	9–12	+++ (all epithelium)	+	9–11	10–11	3–5	3–5	+	-	-	-
Accipitriformes	*Haliaeetus albicilla*	9–10	-	short and wide	14–16	+++ (all epithelium)	+	16–17	12–13	4–7	12–13	+	-	-	-
Accipitriformes	*Pernis apivorus*	2–3–4	+	short and wide	8–10	+++ (all epithelium)	+	8–9	7–8	3–4	3–4	+	-	-	-
Falconiformes	*Falco peregrinus*	6–8	+	short and wide	13–16	+ (basal lamina)	-	6–8	7–8	5–8	5–8	+	-	-	-
Falconiformes	*Falco tinnunculus*	3–4–5	-	long and narrow	14–15	+++ (all epithelium)	-	6–9	7–8	6–10	7–14	+	-	-	-
Strigiformes	*Aegolius funereus*	3–4	-	short and wide	8–11	+++ (all epithelium)	+	9–10	10–11	5–6	7–8	-	+	-	+
Strigiformes	*Athene noctua*	3–5	-	short and wide	8–9	+++ (all epithelium)	+	9–10	8–9	3–4	2–4	-	+	+	-
Strigiformes	*Bubo bubo*	2–4	-	short and wide	6–8	+++ (all epithelium)	+ (adipose cells)	16–17	12–13	10–14	10–14	-	+	-	+
Strigiformes	*Bubo scandiacus*	9–15	-	long and wide	7–9	+++ (all epithelium)	- (adipose cells)	12–13	13–14	9–15	9–15	-	+	-	-
Strigiformes	*Otus megalotis*	2–3	-	short and wide	10–12	+++ (all epithelium)	-	9–10	11–12	4–6	4–6	-	+	-	-
Strigiformes	*Strix aluco*	2–5	-	short and wide	10–12	-	- (numerous blood vessels)	11–12	12–13	5–7	8–9	+	-	+	+
Strigiformes	*Strix uralensis*	3–5	-	short and wide	15–23	-	-	13–14	11–12	3–5	20–22	+	+	-	-
Strigiformes	*Surnia ulula*	6–8	+	long and wide	10–13	+++ (all epithelium)	-	12–13	13–14	12–15	14–16	-	-	+	+
Strigiformes	*Tyto alba*	7–8	-	very short and wide	12–13	+++ (all epithelium)	-	12–13	13–14	16–18	14–18	+	-	-	-

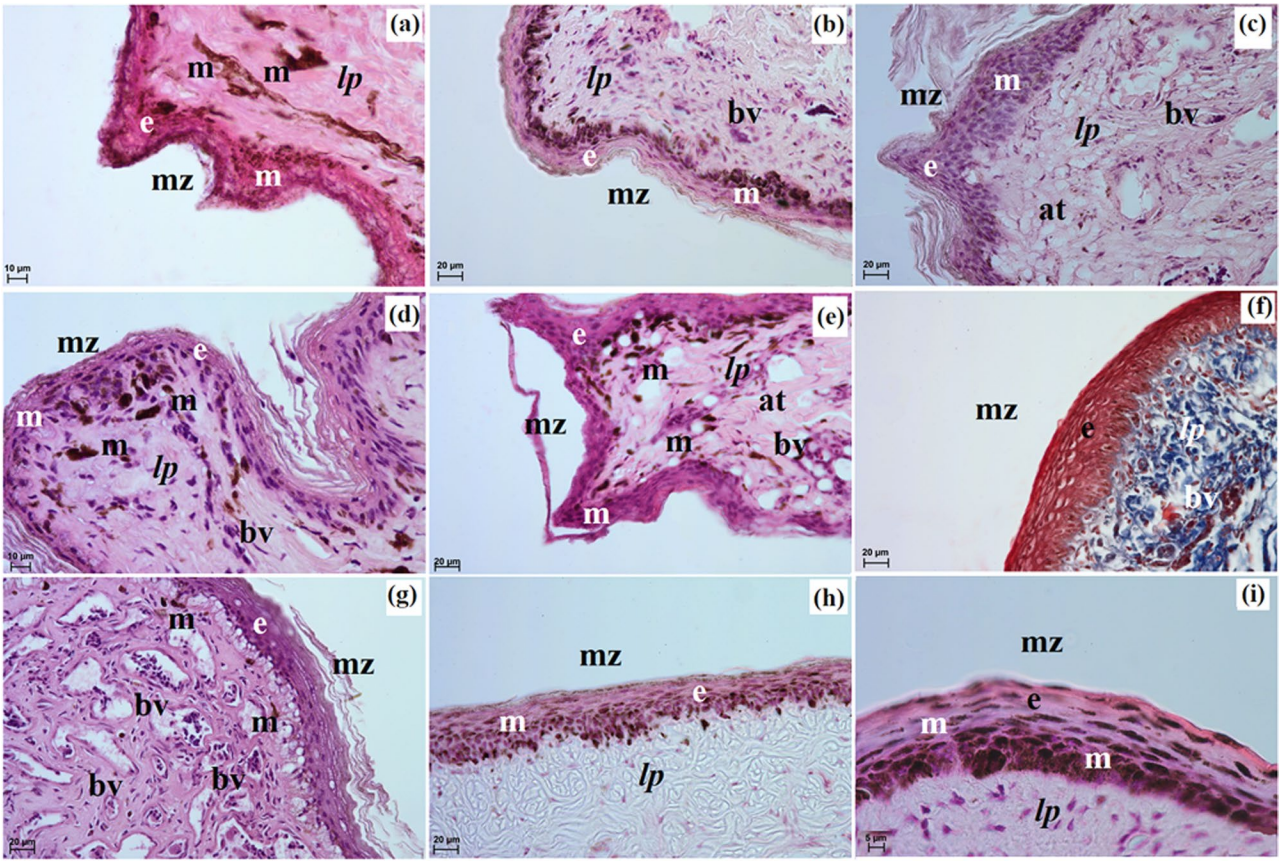

Fig. 7 Differences in the upper and lower eyelids structure between Strigiformes. (**a**) – Boreal Owl; (**b**) – Eurasian Eagle-Owl; (**c**) – Snowy Owl; (**d**) – Little Owl; (**e**) – Northern Hawk Owl; (**f**) – Ural Owl; (**g**) – Tawny Owl; (**h**) – Philippine Scops-Owl; (**i**) – Western Barn Owl. bv – blood vessels, e – epithelium, *lp* – *lamina propria*, m – melanocytes, mz – marginal zone. **a** – e, g – i = Mayer's H&E stain; f = picro-Mallory trichrome stain. Scale bars: **b**, **c**, **e** – h = 20 µm; a = 10 µm; i = 5 µm

all layers of the epithelium in the marginal zone (Fig. 11e and i; Table 2). Moreover, in the Eurasian Sparrowhawk, Steppe Eagle, Common Buzzard, Hen Harrier, White-tailed Eagle, and European Honey-buzzard, numerous melanin clusters surrounding blood vessels were observed in the dense connective tissue of the marginal zone in both eyelids (Fig. 11b, c, e and h; Table 2). In the case of Eurasian Goshawk, Peregrine Falcon, and Eurasian Kestrel, numerous blood vessels were present, but no melanocytes were detected (Fig. 11a, d and i; Table 2).

The structure of the stroma of the upper and lower eyelids was another feature in common for the analyzed representatives of Accipitriformes and Falconiformes. It mainly consists of collagen fibers, but elastic and reticular fibers also were clearly marked. Blood vessels and nerves were present as well as a few clusters of melanocytes and adipocytes (Fig. 11j and s). In most of the analyzed species, very well-developed muscles of eyelids (*m. levator palpebrae dorsalis et m. depressor palpebrae ventralis*) were observed, except for Steppe Eagle and White-tailed Eagle (Fig. 11n and o).

In all analyzed species of Accipitriformes and Falconiformes, the most frequently observed eye-associated lymphoid tissue (EALT) was CALT organized in the form of lymphoid follicle, diffuse lymphocytes and HEV (Fig. 12a and o; Table 2).

Histological analysis of the third eyelid showed the presence of numerous granules of melanin located around blood vessels in the marginal plait of the third eyelid for all examined birds from Accipitriformes and Falconiformes (Fig. 13a and i; Table 3). The eyelid stroma of the examined birds was composed of dense connective tissue with dominant collagen fibers, with clearly marked elastic fibers and reticular fibers, fibrocytes, and regularly arranged clusters of melanocytes located around blood vessels (Fig. 13j and s; Table 3). However, no melanocytes were detected in this structure in the Peregrine Falcon (Table 3).

Another examined feature was the number of thick folds located on the palpebral surface and the number of thin folds on the bulbar surface of the third eyelid in all representatives of Accipitriformes and Falconiformes. A noticeable difference in the number of folds was observed

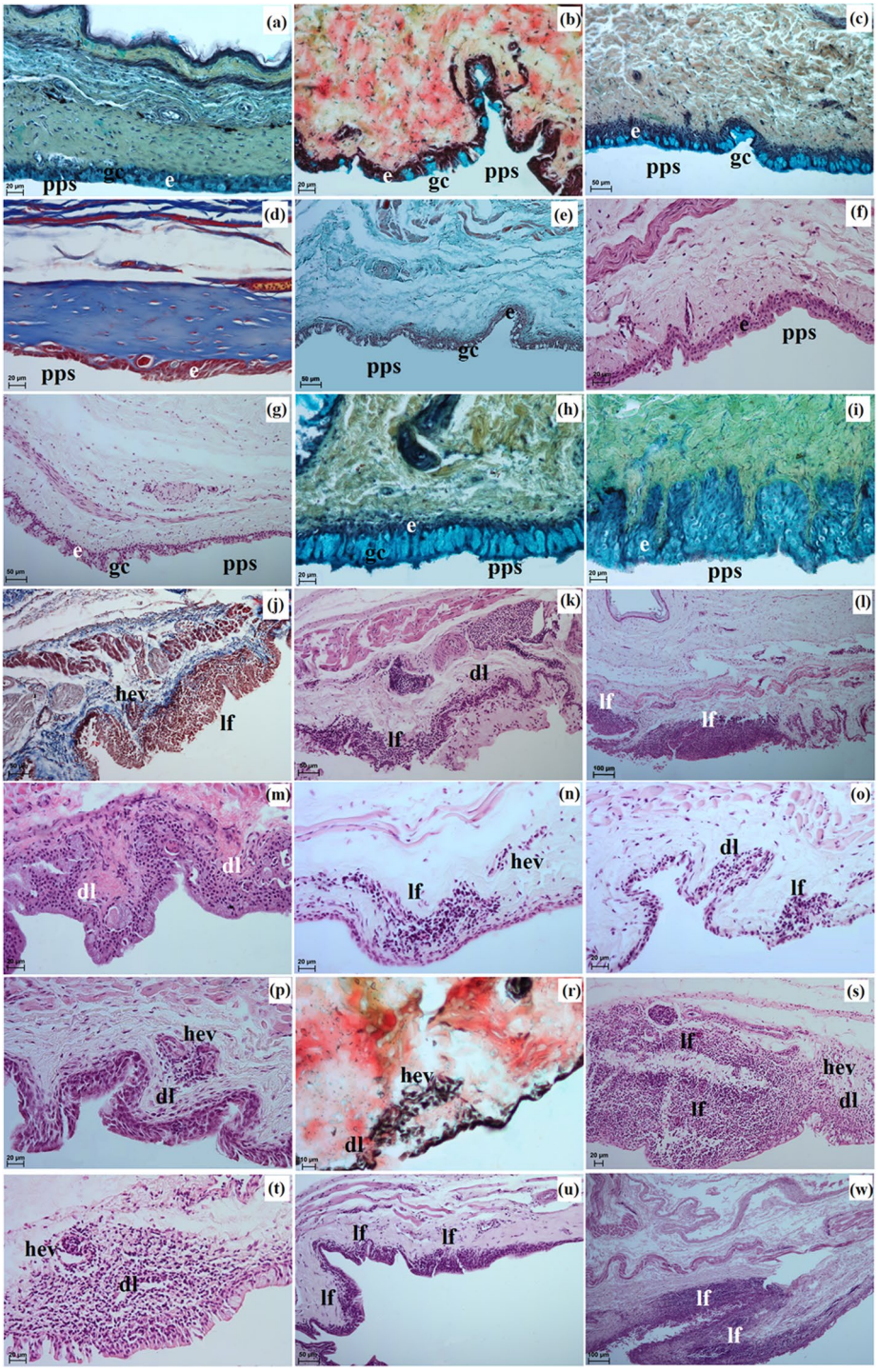

Fig. 8 Comparative structure of upper and lower eyelids in Strigiformes. (**a**), (**p**) and (**r**) – Boreal Owl; (**b**) and (**j**) – Northern Hawk Owl; (**c**), (**n**) and (**o**) – Eurasian Eagle-Owl; (**d**) and (**k**) – Ural Owl; (**e**) and (**s**) – Little Owl; (**f**) and (**t**) – Tawny Owl; (**d**) and (**u**) – Snowy Owl; (**h**) and (**w**) – Philippine Scops-Owl; (**i**), (**l**) and (**m**) – Western Barn Owl. dl – diffuse lymphocytes, hev – high endothelial venules, lf – lymphoid follicle, e – epithelium, gc – goblet cells, pps – posterior palpebral surface. **f**, **g**, **k** – **p**, s – w = Mayer's H&E stain; e = Masson-Goldner trichrome stain; a – **c**, h, i, r = Movat pentachrome (modified Russell-Movat) stain; d, j = picro-Mallory trichrome stain. Scale bar: l, w = 100 µm; **c**, **e**, **g**, **j**, **k**, u = 50 µm; **a**, **b**, **d**, **f**, **h**, **i**, **m** – **p**, **s**, t = 20 µm; d, *r* = 10

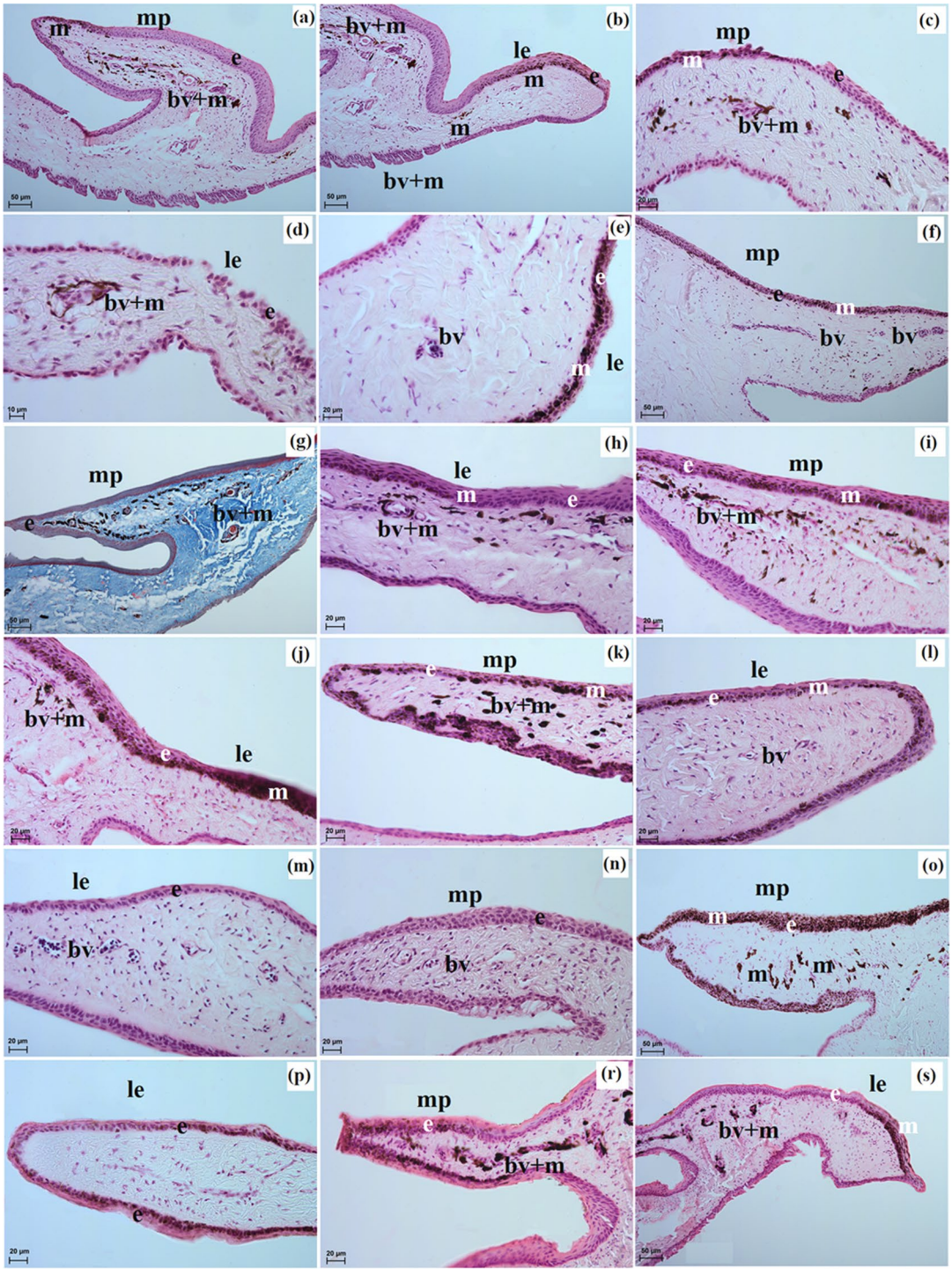

Fig. 9 Differences in the morphology of the third eyelid structure among Strigiformes. (**a**) and (**b**) – Boreal Owl; (**c**) and (**d**) – Northern Hawk Owl; (**e**) and (**f**) – Eurasian Eagle-Owl; (**g**) and (**h**) – Ural Owl; (**i**) and (**j**) – Little Owl; (**k**) and (**l**) – Tawny Owl; (**m**) and (**n**) – Snowy Owl; (**o**) and (**p**) – Philippine Scops-Owl; (**r**) and (**s**) – Western Barn Owl. e – epithelium, bv + m – blood vessels and melanocytes, le – leading edge, m – melanocytes, mp – marginal plait. **a** – **f**, **h** – s = Mayer's H&E stain; g = picro-Mallory trichrome stain. Scale bars: **a**, **b**, **f**, **g**, **o**, **s** = 50 µm; **c**, **e**, **h**, **i** – **n**, **p**, *r* = 20 µm; d = 10 µm

between each examined species (Table 3). The smallest number of palpebral folds (from 6 to 11) was found in the Eurasian Goshawk, Eurasian Sparrowhawk and Common Buzzard, while the largest number of these folds (from 21 to 30) was observed in the Steppe Eagle, White-tailed Eagle and Peregrine Falcon (Table 3). The number of folds on the bulbar surface in all examined birds from both orders was consistently higher (from 23 to 45) than

Table 3 Characteristic features of the structure of third eyelids in the examined birds. (+) – presence, (-) – absence

Order	Species	Marginal plait and leading edge	Melanocytes in the marginal plait of stroma	Melanocytes in the marginal plait epithelium	Layers of the nucleated cells in the marginal plait and leading edge	Layers of the nucleated cells on the palpebral surface	Layers of the nucleated cells on the bulbar surface	Stroma of the third eyelid						Numbers of the palpebral folds	Numbers of the conjunctival folds
								collagen fibres	elastic fibres	reticular fibres	fibrocytes	blood vessels	melanocytes		
Accipitriformes	*Accipiter gentilis*	thick	+	-	5–6	5–6	3–4	+	+	+	+	+	+	6–9	29–31
Accipitriformes	*Accipiter nisus*	thick	+	+	5–6	3–4	3–4	+	+	+	+	+	+	9–10	29–30
Accipitriformes	*Aquila nipalensis*	thick	+	+	12–17	3–6	10–13	+	+	+	+	+	+	9–11	41–45
Accipitriformes	*Buteo buteo*	thick	+	+	5–6	4–5	2–4	+	+	+	+	+	+	16–17	41–43
Accipitriformes	*Circus cyaneus*	thick	+	+	5–7	2–3	2–3	+	+	+	+	+	+	15–16	41–43
Accipitriformes	*Haliaeetus albicilla*	thick	+	-	7–8	6–10	11–13	+	+	+	+	+	+	24–29	29–30
Accipitriformes	*Pernis apivorus*	thick	+	+	5–7	3–6	3–4	+	+	+	+	+	+	29–30	32–33
Falconiformes	*Falco peregrinus*	thin	+	+	3–5	3–5	3–4	+	+	+	+	+	-	21–23	31–33
Falconiformes	*Falco tinnunculus*	thin	+	-	3–5	3–6	3–4	+	+	+	+	+	+	10–14	23–28
Strigiformes	*Aegolius funereus*	very thick	+	+	7–8	5–6	10–13	+	+	+	+	+	-	10–13	29–31
Strigiformes	*Athene noctua*	very thick	+	+	9–12	8–10	2–4	+	+	+	+	+	-	24–25	30–31
Strigiformes	*Bubo bubo*	very thick	+	+	5–6	4–5	3–4	+	+	+	+	+	-	10–23	29–30
Strigiformes	*Bubo scandiacus*	very thick	-	-	6–9	2–3	3–4	+	+	+	+	+	-	32–34	65–70
Strigiformes	*Otus megalotis*	very thick	+	+	3–9	2–3	3–4	+	+	+	+	+	-	11–12	30–31
Strigiformes	*Strix aluco*	very thick	+	+	8–10	8–11	4–5	+	+	+	+	+	-	22–24	31–32
Strigiformes	*Strix uralensis*	very thick	+	+	3–8	2–6	3–5	+	+	+	+	+	-	14–16	20–21
Strigiformes	*Surnia ulula*	very thick	+	+	5–6	4–5	2–4	+	+	+	+	+	+	17–25	31–40
Strigiformes	*Tyto alba*	very thick	+	+	8–9	7–8	3–4	+	+	+	+	+	+	16–17	42–64

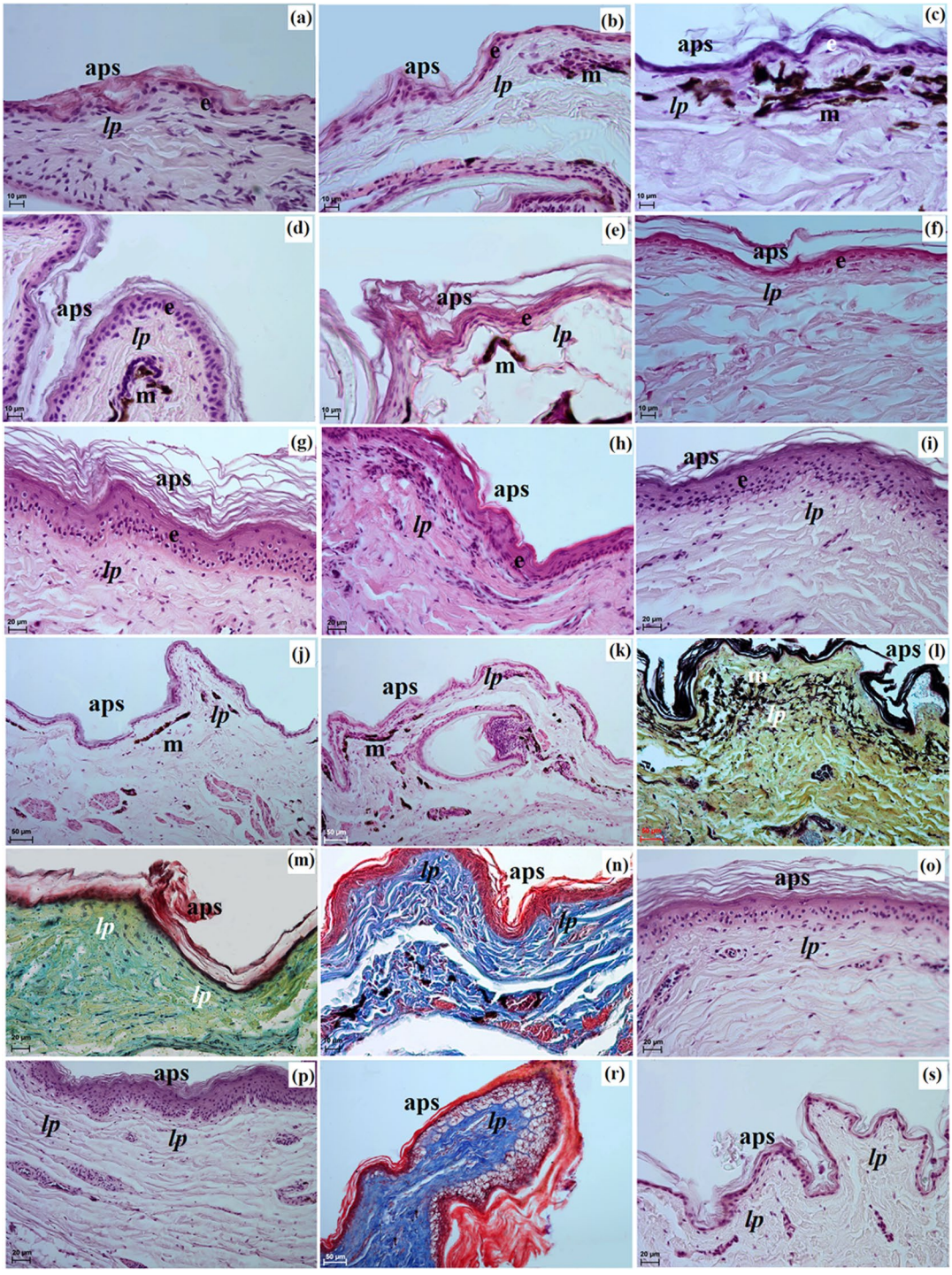

Fig. 10 Shared structural characteristics of the upper and lower eyelids in Accipitriformes and Falconiformes. (**a**) and (**m**) – Eurasian Goshawk; (**b**) and (**k**) – Hen Harrier; (**c**) and (**l**) – European Honey-buzzard; (**d**) and (**n**) – Eurasian Sparrowhawk; (**e**) and (**j**) – Common Buzzard; (**f**) and (**r**) – Eurasian Kestrel; (**g**) and (**o**) – Steppe Eagle; (**h**) and (**s**) – Peregrine Falcon; (**i**) and (**p**) – White-tailed Eagle. aps – anetrior palpebral surface, e – epithelium, *lp* – *lamina propria*, m – melanocytes. a – **k**, **o**, **p**, s = Mayer's H&E stain; **l**, m = Movat pentachrome (modified Russell-Movat) stain; n, r = picro-Mallory trichrome stain. Scale bars: j – **l**, *r* = 50 µm; g – **i**, **m**, **o**, **p**, s = 20 µm; **a**– **f**, *n* = 10 µm

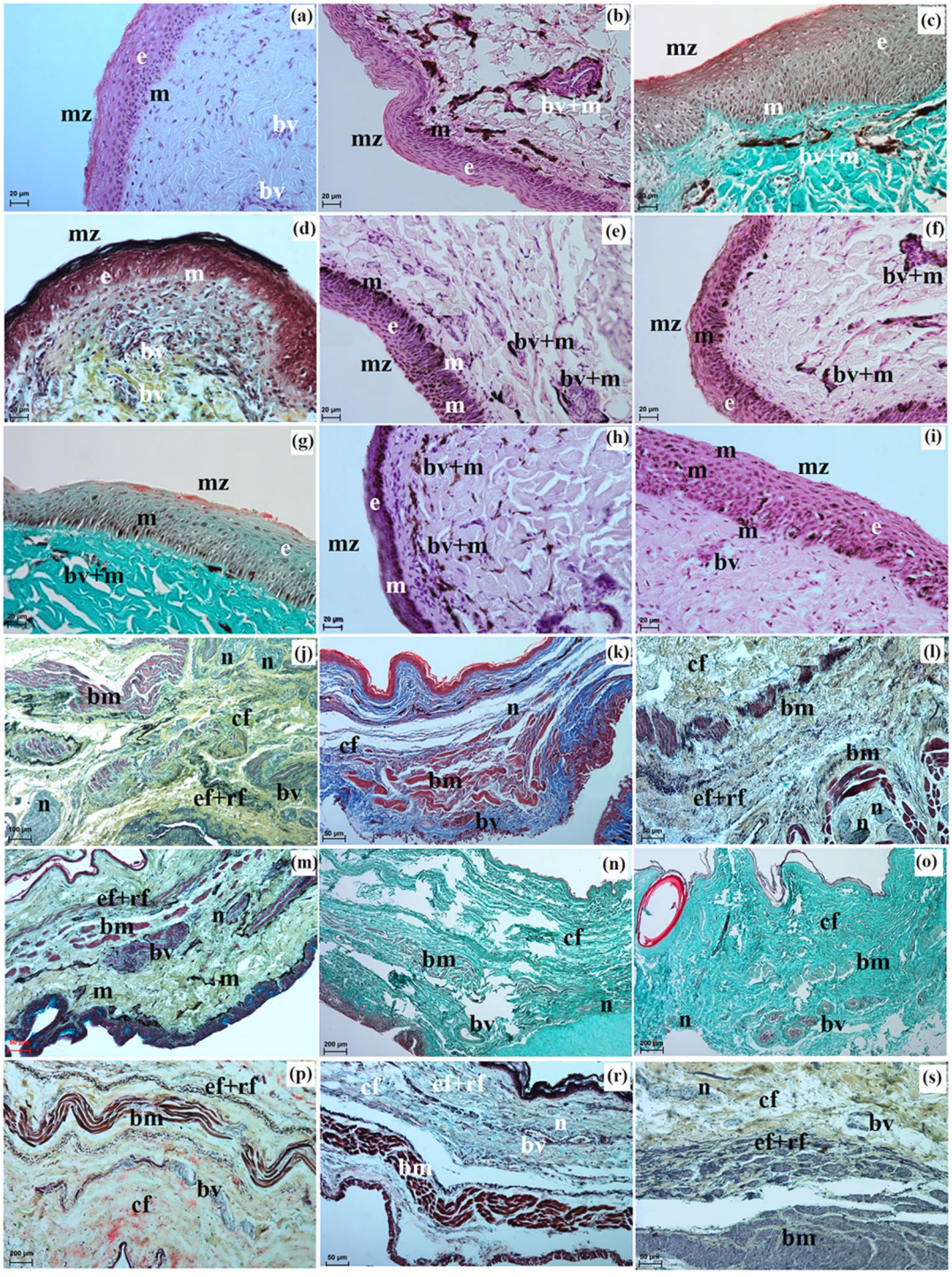

Fig. 11 Common traits of the upper and lower eyelids in Accipitriformes and Falconiformes. (**a**) and (**j**) – Eurasian Goshawk; (**b**) and (**l**) – Common Buzzard; (**c**) and (**n**) – Steppe Eagle; (**d**) and (**r**) – Peregrine Falcon; (**e**) and (**k**) – Eurasian Sparrowhawk; (**f**) and (**m**) – Hen Harrier; (**g**) and (o) – White-tailed Eagle; (**h**) and (**p**) – European Honey-buzzard; (**i**) and (**s**) – Eurasian Kestrel. bm – bundles of muscle, bv – blood vessels, bv + m – blood vessels and melanocytes, cf. – collagen fibres, e – epithelium, ef + rf – elastic fibres and reticular fibres, m – melanocytes, mz – marginal zone, n – nerves. **a**, **b**, **e**, **f**, **h**, **i** = Mayer's H&E stain; **c**, **g**, **n**, **o** = Masson Goldner trichrome stain; **d**, **j**, **l**, **m**, **p** – **s** = Movat pentachrome (modified Russell-Movat) stain; k = picro-Mallory trichrome stain. Scale bars: **n**, **o**, *p* = 200 µm; **j** = 100 µm; **k**, **l**, **m**, **r**, **s** = 50 µm; **a** – **i** = 20 µ**m**

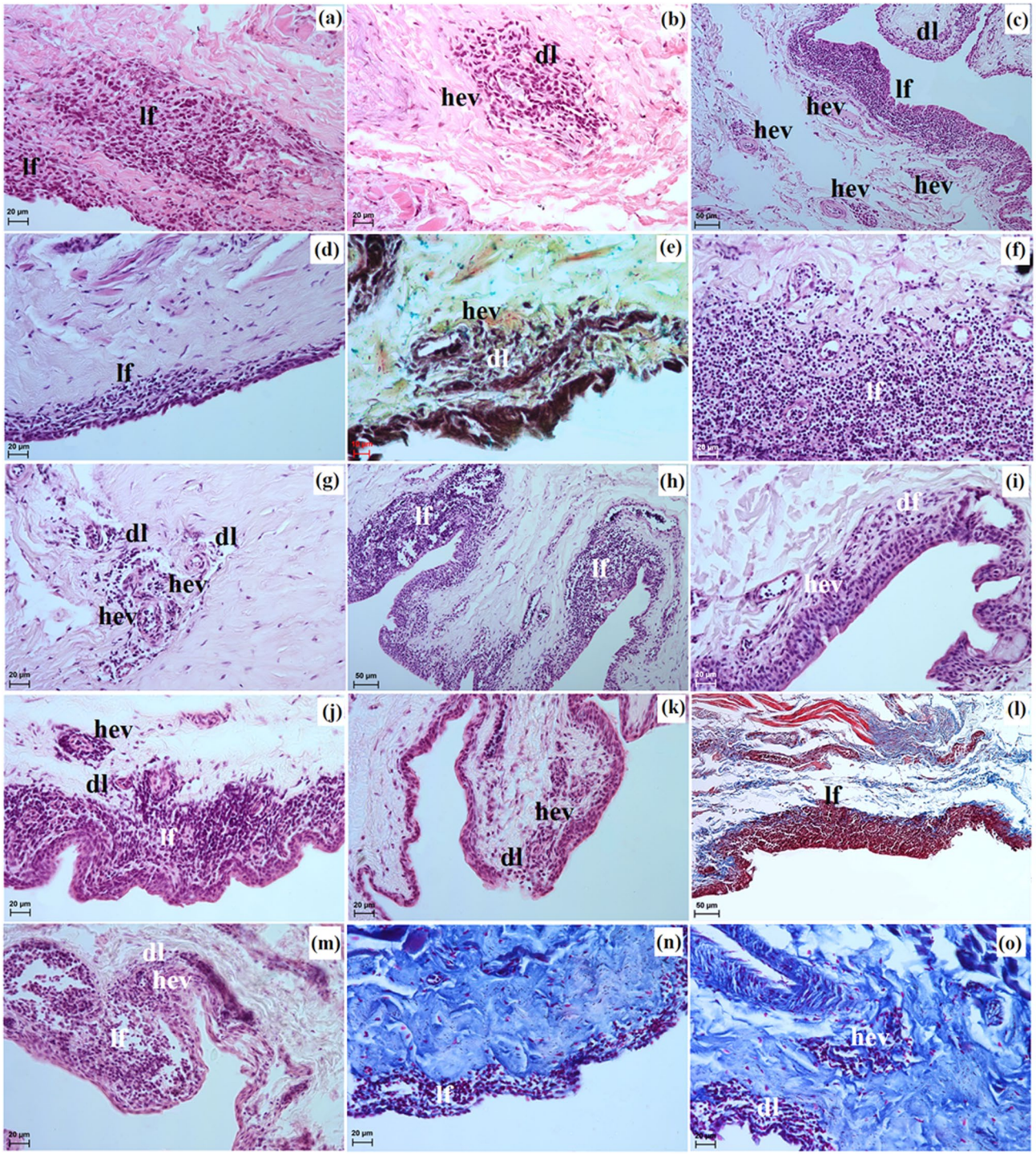

Fig. 12 Structural similarities of the upper and lower eyelids in Accipitriformes and Falconiformes. (**a**) and (**b**) – Eurasian Goshawk; (**c**) – Common Buzzard; (**d**) and (**e**) – European Honey-buzzard; (**f**) and (**g**) – Steppe Eagle; (**h**) and (**i**) – White-tailed Eagle; (**j**) – Peregrine Falcon; (k) and (**l**) – Hen Harrier; (**m**) – Eurasian Kestrel; (**n**) and (**o**) – Eurasian Sparrowhawk. dl – diffuse lymphocytes, hev – high endothelial venules, lf – lymphoid follicle. **a** – **d**, **f** – **k**, **m** = Mayer's H&E stain; e = Movat pentachrome (modified Russell-Movat) stain; l, n, o = picro-Mallory trichrome stain. Scale bars: c, h, l, = 50 µm; a, b, d, f – g, i – k, m – o = 20 µm; e = 10 µm

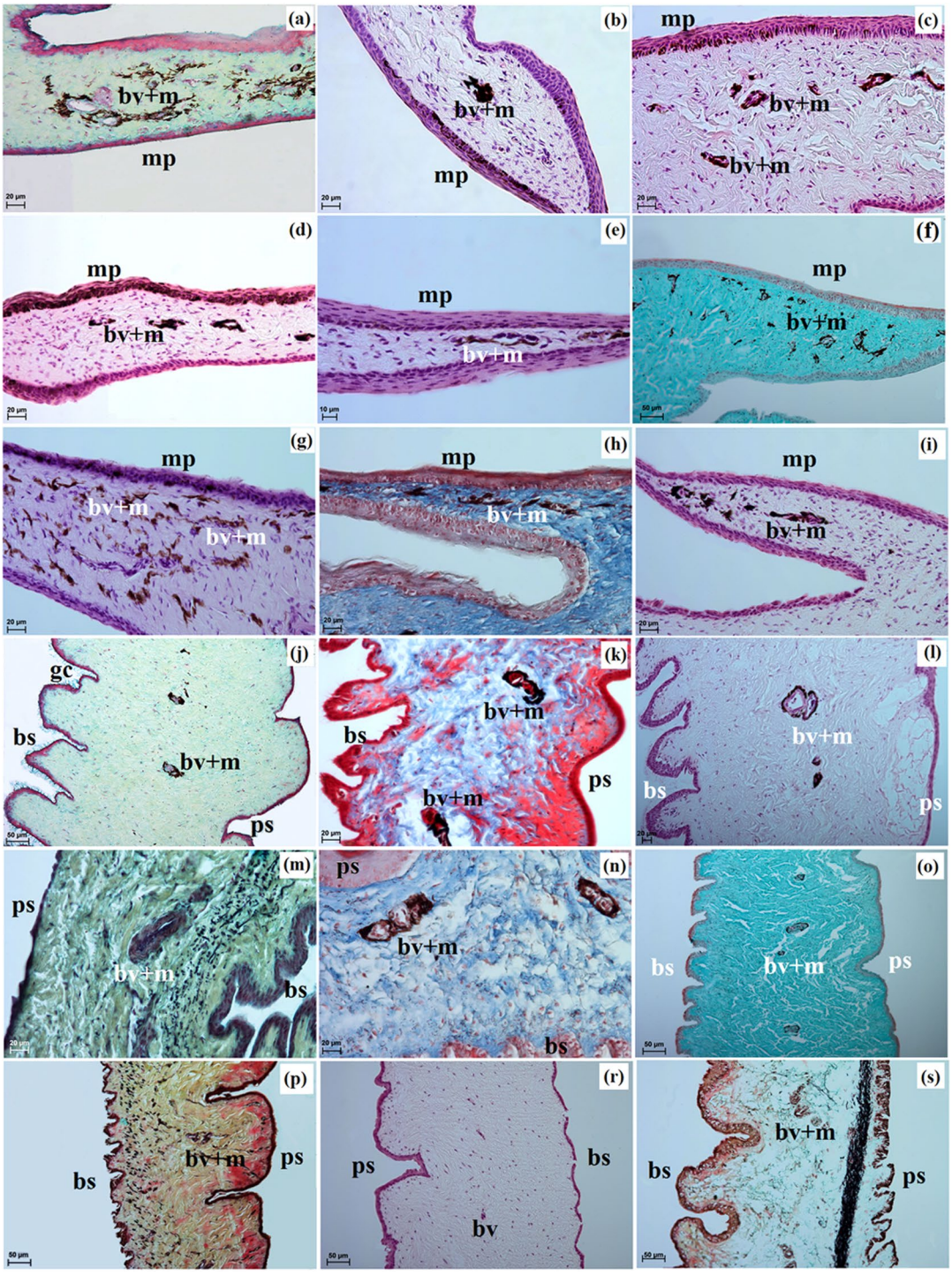

Fig. 13 Shared structural characteristics of the third eyelid in Accipitriformes and Falconiformes. (**a**) and (**j**) – Eurasian Goshawk; (**b**) and (**k**) – Eurasian Sparrowhawk; (**c**) and (**l**) – Common Buzzard; (**d**) and (**m**) – Hen Harrier; (**e**) and (**n**) – Steppe Eagle; (**f**) and (**o**) – White-tailed Eagle; (**g**) and (**p**) – European Honey-buzzard; (**h**) and (**r**) – Peregrine Falcon, (**i**) and (**s**) – Eurasian Kestrel. bs – bulbar surface, bv – blood vessels, bv + m – blood vessels and melanocytes, mp – marginal plait, ps – palpebral surface. **b** – **e**, **g**, **i**, **l**, r = Mayer's H&E stain; **f**, o = Masson-Goldner trichrome stain; **d**, **j**, **m**, **p**, s = Movat pentachrome (modified Russell-Movat) stain; **h**, **k**, n = picro-Mallory trichrome stain. Scale bars: **f**, **j**, **o**, **p** – s = 50 µm; a – d, g – **i**, **k** – *n* = 20 µm; e = 10 µm

on the palpebral surface (from 6 to 30) (Table 3). The highest number of bulbar folds (from 41 to 45) was found in the Common Buzzard, Hen Harrier and European Honey-buzzard (Table 3).

The greatest differentiation between the analyzed bird species within Accipitriformes and Falconiformes orders was observed in the folds of skin located on the anterior surface of the upper and lower eyelids. The most common short and wide folds of skin were present in the Eurasian Sparrowhawk, Steppe Eagle, Peregrine Falcon, White-tailed Eagle and European Honey-buzzard (Fig. 14a and e; Table 2). While long and narrow skin folds occurred in Eurasian Goshawk, Common Buzzard, and Eurasian Kestrel (Fig. 14f and i; Table 2). Significant differences were also observed in the number of cell layers forming the stratified columnar epithelium in the marginal zone in both eyelids. In the Eurasian Sparrowhawk, Hen Harrier and European Honey-buzzard, the number of layers of cells ranged from 8 to 12, while in the remaining species from these orders the number of cell layers was between 13 and 20 (Fig. 11a and i; Table 2). Differences were also observed in the length and width of the tarsal plate. In the Peregrine Falcon and Eurasian Kestrel these dimensions were the smallest compared to Accipitriformes. However, large differences in length and width of the tarsal plate were observed within Accipitriformes. The largest tarsal plate was found in the Steppe Eagle and White-tailed Eagle, while the smallest tarsal plate was observed in the Eurasian Goshawk and European Honey-buzzard (Table 2). Another feature that significantly varied between birds representing both orders was the number of cell layers forming the posterior epithelium in the ocular zone of the upper eyelids and in the plate zone and ocular zone of the lower eyelids. In the bulbar zone of the upper eyelid, it was from 3 to 5 layers of nucleated cells in the Eurasian Sparrowhawk, Common Buzzard, Hen Harrier and European Honey-buzzard, and from 4 to 5 to 10 layers of the cells in the Eurasian Goshawk, Steppe Eagle, White-tailed Eagle, Peregrine Falcon, and Eurasian Kestrel (Fig. 14j and s; Table 2). In the plate zone and bulbar zone in the lower eyelid were observed from 3 to 4 to 4–5 layers of the cells in the Eurasian Goshawk, Eurasian Sparrowhawk, Hen Harrier, and European Honey-buzzard. Higher numbers of cells layers (5–14) was observed in the Common Buzzard, Steppe Eagle, White-tailed Eagle, Peregrine Falcon and Eurasian Kestrel (Table 2).

Morphological analysis of the third eyelid showed that the marginal plait and the leading edge in the Falconiformes were thin (Fig. 15a and b) contrary to Accipitriformes where they were thick (Fig. 15c and i). The presence of granules of melanin was demonstrated on the marginal plait epithelium in the Eurasian Sparrowhawk, Steppe Eagle, Common Buzzard, Hen Harrier, Peregrine Falcon and European Honey-buzzard (Fig. 15j, m, o, r and s; Table 3). Moreover, it was found that the number of layers of nucleated cells forming non-keratinized stratified squamous epithelium in the marginal plait and the leading edge varies in the Falconiformes from 3 to 5 layers, while in the Accipitriformes from 5 to 8 layers (Eurasian Goshawk, Eurasian Sparrowhawk, Common Buzzard, Hen Harrier, White-tailed Eagle and European Honey-buzzard) and as far as 12 to 17 layers in Steppe Eagle (Fig. 15j and s; Table 3). It was also observed that in Falconiformes the number of the layers of cells forming the palpebral and bulbar surface of the third eyelid is very similar between the analyzed species and it ranges from 3 to 6 layers of nucleated cells (Fig. 16a and d; Table 3). However, in representatives of Accipitriformes, great diversity in the number of cell layers was found. In the Steppe Eagle and White-tailed Eagle, the number of layers of cells ranged from 6 to 13 (Fig. 16e and h; Table 3), while in other Accipitriformes species it ranged from 2 to 3 to 6 (Fig. 17a and j; Table 3). Moreover, it was observed that in Falconiformes the third eyelid was thin (Fig. 15a and b), in contrast to Accipitrformes where it was much thicker, especially in the Steppe Eagle and White-tailed Eagle (Fig. 15c and i).

Discussion

Owls have always been one of the contentious groups of predatorial birds and many authors do not associate them with raptors [31, 38, 50, 54]. However, due to the monophyletic raptorial landbirds lineage of the order Strigiformes we include owls in the raptor group, as some other authors do [34, 39, 52, 59–61]. For many researchers, the key distinguishing factor between species from Accipitriformes, Falconiformes and Strigiformes was the time of the day at which they are active [5, 7, 62, 63]. All analyzed representatives Accipitriformes and Falconiformes (Table 1) are described as diurnal [62, 63]. Owls are generally considered nocturnal [64], but out of all the species representing Strigiformes studied in this research, only the Tawny Owl and Philippine Scops-Owl may be regarded as active strictly during the night [64, 65]. However, the Tawny Owl is chiefly nocturnal but occasionally can be diurnal [66]. The Western Barn Owl and Ural Owl, reported as mainly nocturnal, sometimes are also crepuscular [67, 68]. The Little Owl is essentially hunting nocturnal and crepuscular, but can also be diurnal [69]. The Northern Hawk Owl is especially diurnal and will hunt in the bright sunlight [70], however in its northern latitudes it will hunt any time of day except severe weather conditions [72] similarly to other species living in northern regions without summer darkness (like Boreal Owl and Eurasian Eagle-Owl) [64, 71] or in circumpolar range (like Snowy Owl) [72].

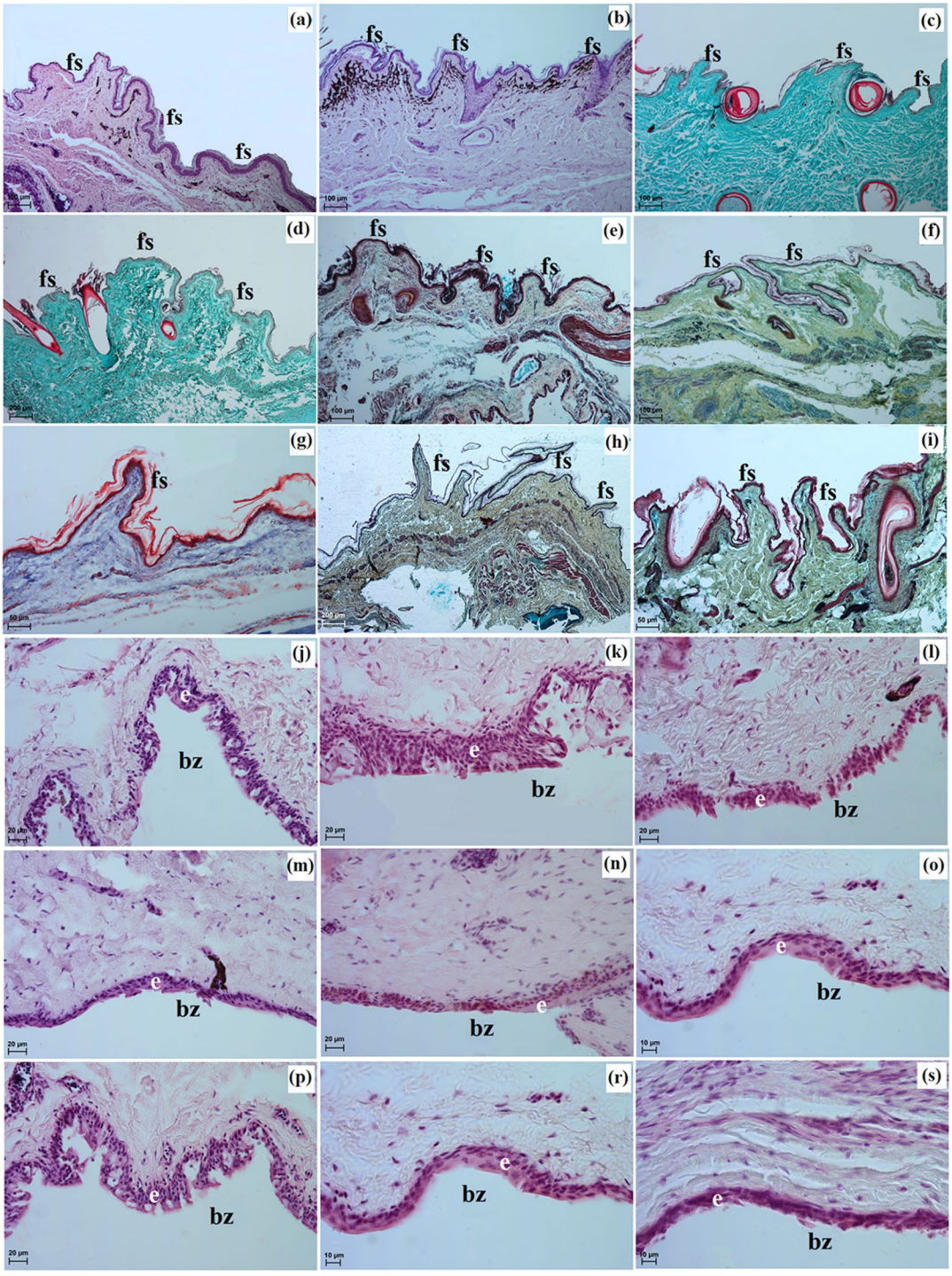

Fig. 14 Differences in the upper eyelid and lower eyelids structure between Accipitriformes and Falconiformes. (**a**) and (**j**) – Eurasian Sparrowhawk; (**b**) and (**m**) – European Honey-buzzard; (**c**) and (**o**) – Steppe Eagle; (**d**) and (**p**) – White-tailed Eagle; (**e**) and (**r**) – Peregrine Falcon; (**f**) and (**m**) – Eurasian Goshawk; (**g**) and (**s**) – Eurasian Kestrel; (**h**) and (**k**) – Common Buzzard; (**i**) and (**l**) – Hen Harrier. bz – bulbar zone, e – epithelium, fs – folds of skin. **a**, **b**, **j** – **s** = Mayer's H&E stain; c, d = Masson-Goldner trichrome stain; **e**, **f**, **h**, **i** = Movat pentachrome (modified Russell-Movat) stain; g = picro-Mallory trichrome stain. Scale bars: d, h = 200 µm; a – **c**, e, f = 100 µm; **g**, **i** = 50 µ**m**; j – **n**, **o** – s = 20 µm

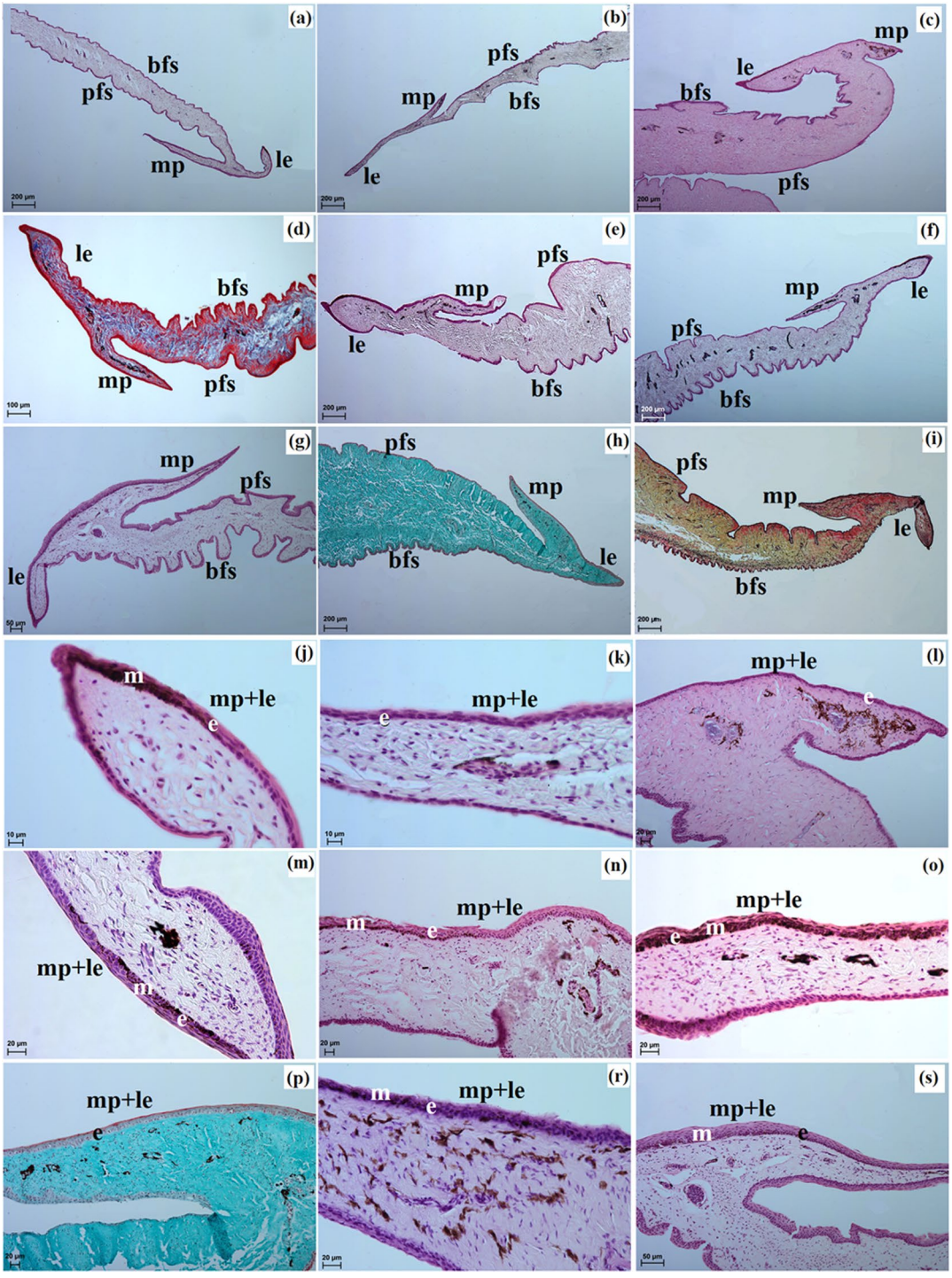

Fig. 15 Comparison of the third eyelid structure in Accipitriformes and Falconiformes. (**a**) and (**j**) – Peregrine Falcon; (**b**) and (**k**) – Eurasian Kestrel; (**c**) and (**l**) – Eurasian Goshawk; (**d**) and (**m**) – Eurasian Sparrowhawk; (**e**) and (**n**) – Common Buzzard; (**f**) and (**o**) – Hen Harrier; (**g**) and (**s**) – Steppe Eagle; (**h**) and (**p**) – White-tailed Eagle; (**i**) and (**r**) – European Honey-buzzard. bfs – bulbar folds, **e** – epithelium, **m** – melanocytes, mp – marginal plait, le – leading edge, pfs – palpebral folds. **a** – **c**, **e** – **g**, **j** – **o**, **r**, s = Mayer's H&E stain; d – pico-Mallory trichrome stain; h, p = Masson-Goldner trichrome; i = Movat pentachrome (modified Russell-Movat) stain. Scale bars: **a** – **c**, **e**, **f**, **h**, **i** = 200 µm; **d** = 100 µm; **g**, **s** = 50 µm; **l** – *r* = 20 µm; **j**, **k** = 10 µm

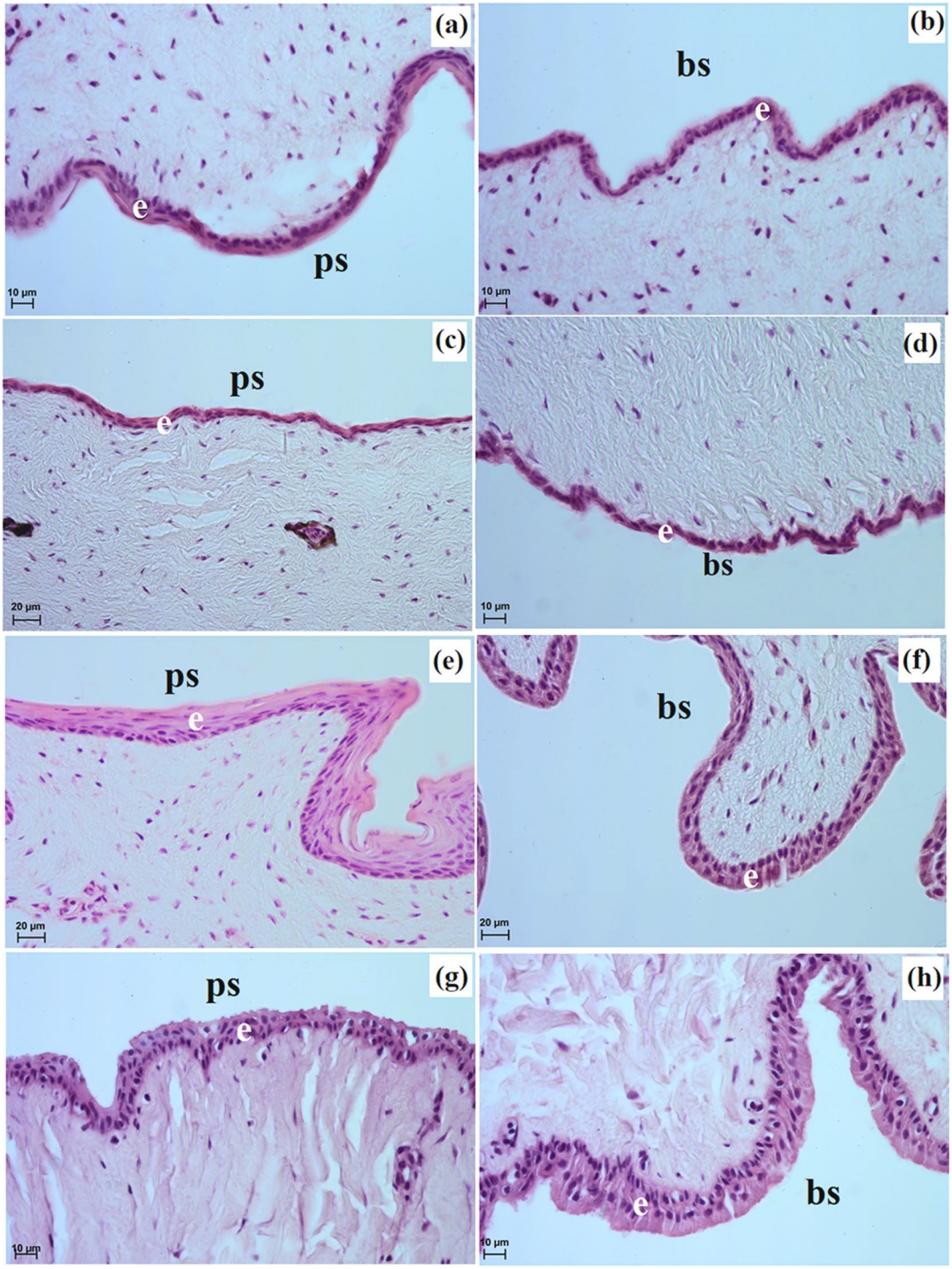

Fig. 16 Differences in the third eyelid structure between Accipitriformes and Falconiformes. (**a**) and (**b**) – Peregrine Falcon; (**c**) and (**d**) – Eurasian Kestrel; (**e**) and (**f**) – Steppe Eagle; (**g**) and (**h**) –White-tailed Eagle. e – epithelium, bs – bulbar surface, ps – palpebral surface. a – h = Mayer's H&E stain. Scale bars: c, e, f = 20 µm; **a**, **b**, **d**, **g**, **h** = 10 µm

Due to the common evolutionary history of Strigiformes, Accipitriformes, and Falconiformes, and the fact that many species of owls can be characterized by the same activity as typical diurnal predators, we wanted to determine which features of the eyelid structures are common for representatives of these three orders and can be considered as derived from a common ancestor and what features could be adaptations to hunting in specific groups of birds.

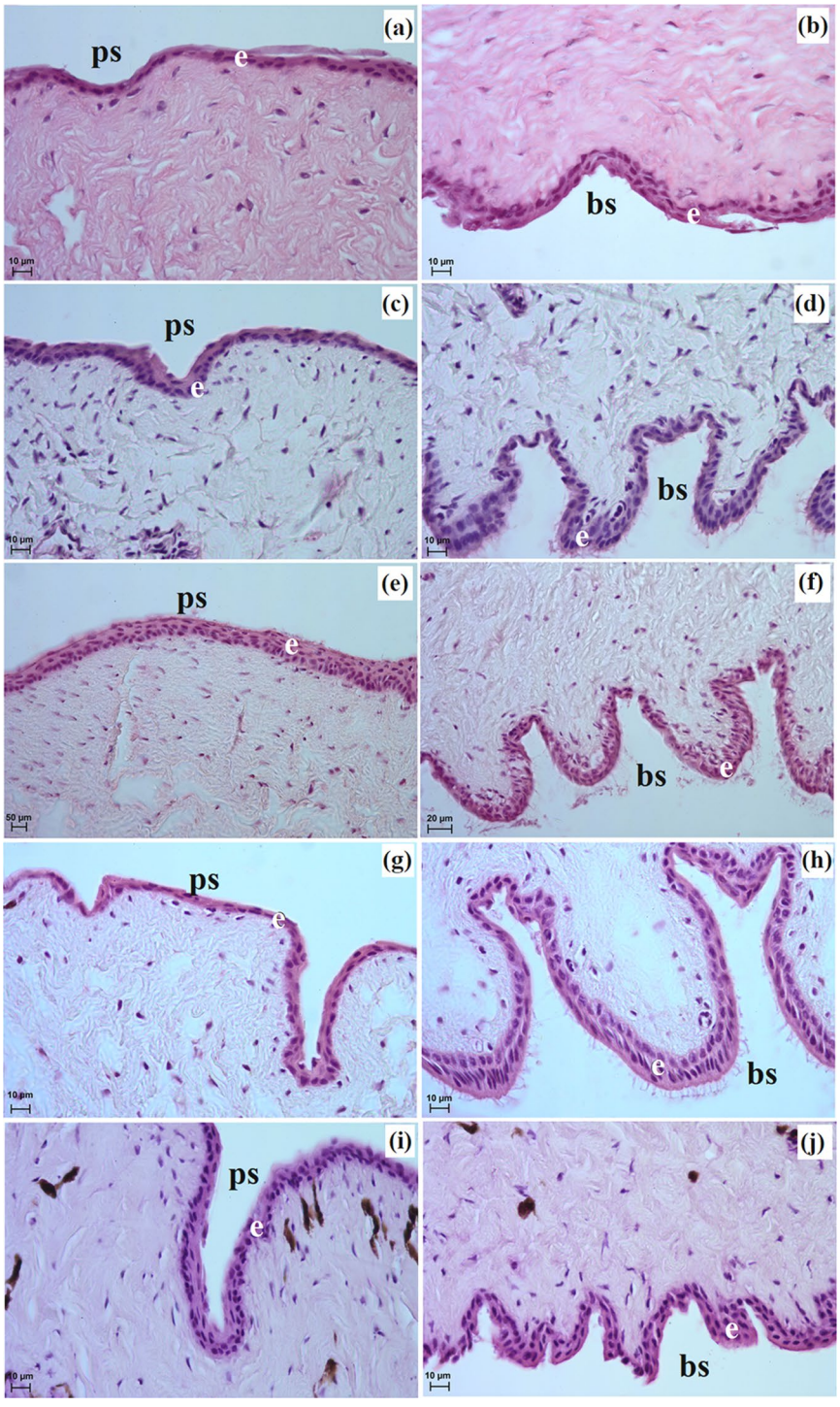

Fig. 17 Comparative analysis of the third eyelid structure in Accipitriformes and Falconiformes. (**a**) and (**b**) – Eurasian Goshawk; (**c**) and (d) – Eurasian Sparrowhawk; (**e**) and (**f**) – Common Buzzard; (**g**) and (**h**) – Hen Harrier; (**i**) and (**j**) – European Honey-buzzard. e – epithelium, bs – bulbar surface, ps – palpebral surface. a – j = Mayer's H&E stain. Scale bars: e = 50 µm; f = 20 µm; a – d, g – j = 10 µm

Our histological examinations of the eyelids have shown that there are several common features in their structure in Strigiformes, Accipitriformes and Falconiformes. These features are: (1) the type of epithelium covering the anterior and posterior palpebral surface and the marginal zone of both eyelids, (2) the presence of folds of skin and folds of the conjunctiva, (3) the presence of sweat and sebaceous glands, (4) a stroma structure of the eyelids and (5) the presence of a tarsal plate in the lower eyelid. Similarly, in the histology of the third eyelid,

features common for representatives of all three examined orders of birds can also be distinguished. They are: (1) the presence of the same type of epithelium covering the marginal plait and the leading edge and the palpebral surface and bulbar surface, (2) the presence of palpebral folds and bulbar folds, (3) the structure of the stroma of the third eyelid, which is made of collagen fibers, elastic and reticular fibers with a regular arrangement. These features constitute the basic histological structure of the eyelids [21] and they are also observed in representatives of other orders of birds so it can be assumed that these features are inherited from a common ancestor [11–14, 20]. Another characteristic observed in all of the examined species was a different number of cell layers covering the palpebral surface and the bulbar surface. These observations are consistent with results obtained for other birds [9, 11–14, 16, 20] and indicate that these may be either species specific or individual features.

Despite the observed similarities in the anatomy of the eyelids between the analyzed bird orders, owls turned out to be a rather distinct and internally diverse group. The morphology of the eyelids in Strigiformes is specific and the upper eyelid is larger and more mobile, contrary to typical diurnal birds of prey [11, 21–23]. Our studies revealed large variations in the histology of the upper and lower eyelids in the examined owls. The primary differences were the different number of cell layers forming the epithelium of the anterior and posterior palpebral surface and marginal zone of both eyelids, which, due to the fact that it consists of specialized types of skin cells (stem cells), is intended to maintain the integrity of the skin during homeostasis but also has an impact on the protection of deeper structures against harmful external factors and mechanical injuries [73]. Moreover, differences in sizes and thickness of folds of skin in the eyelids were found. Short and wide folds of skin in both eyelids dominated in owls. Due to the characteristic shape of the eyeball and its location in the skull, this may indicate that these folds have important protective functions for the eyeball in owls. However, short and wide folds of skin can be found also in the upper and lower eyelids of some Accipitriformes (e.g., Eurasian Sparrowhawk, Steppe Eagle, European Honey-buzzard) and in the Peregrine Falcon (Order: Falconiformes). These birds have different strategies for catching prey and their circadian activity is different, so it can be assumed that the size and shape of these folds are not connected with hunting adaptation. Interestingly, Western Barn Owl was the only species characterized by the presence of skin folds resembling bricks.

The presence of a characteristic structure in the form of a large adipose pad in the upper and lower eyelids was demonstrated for almost all analyzed Strigiformes species with the exception of the Little Owl. The presence of adipose pads in eyelids was confirmed for several different species of birds [11, 14]. Large adipose pads in eyelids were observed in Great Cormorant (*Phalacrocorax carbo*), African Penguin (*Spheniscus demersus*) and Pied Avocet (*Recurvirostra avosetta*) [14]. However, this structure is absent for example in Luzon Bleeding-heart (*Gallicolumba luzonica*) and Greater Flamingo (*Phoenicopterus roseus*) [14]. The role of this structure is not clear. However, it does not seem to be strictly related only to one environment, e.g. aquatic, because it is not found in all water birds examined so far. It tends to be present in species that are exposed to cold e.g. diving in cold water (e.g., Great Cormorant and African Pengiun) or migrating flying at high altitudes and absent in species from warm environments (e.g., Greater Flamingo). This may indicate that maybe the adipose pad's role in the eyelids is to protect the eyeball against the cold. This would also be confirmed by the lack of adipose pads in the eyelids in the Little Owl. This species does not occur in very cold regions [69]. Interestingly, the adipose pad is present in the eyelids of the Philippine Scops-Owl, which inhabits a warm tropical climate. However, it is worth noting that this species occurs at high altitudes and has been recorded in pine forests of Luzon at elevations of 2,200–2,300 m, where temperatures are lower [74, 75]. However, further research is necessary to fully understand the role of this structure.

Differences were also observed in the size of the tarsal plate located in the lower eyelid. The largest tarsal plates were observed in the Eurasian Eagle-Owl and Ural Owl. According to Jochems and Phillips [11], this tarsal plate in Barred Owl (*Strix varia*) was also large. Moreover, a big tarsal plate in lower eyelid was also reported for Greater Rhea (*Rhea americana*), while in the Luzon Bleeding-heart this structure was poorly marked [14]. Therefore, it seems that the size of the tarsal plate in the lower eyelid may be related to the size of the skull and eye socket. Consequently, it should be proportionally bigger in larger bird species.

Moreover, in the marginal zone of both eyelids in Boreal Owl and Eurasian Eagle-Owl large accumulations of clusters of melanocytes were observed. The role of this structure is not clear, however, both species hunt primarily after dark except in the northern range of their distribution [64, 76]. Presumably, their eyes need additional protection against the increased amount of daylight in regions without summer darkness.

Interestingly, the presence of adipose cells in the marginal zone of both eyelids was found only in the Snowy Owl and Northern Hawk Owl. Both species are circumpolar and drop through the snow to catch prey in winter hunts [70, 72] so the presence of fat cells in the edge of the eyelid may give additional protection for the eye against the cold.

The third eyelid of Strigiformes was opaque white [77, 78] while in other species it was rather transparent or translucent. The third eyelid in the owls was very thick contrary to Accipitriformes and Falconiformes (especially the thin third eyelid along with a marginal plait in Peregrine Falcon and Eurasian Kestrel). These observations consist with the fact that the eyes of predatory birds need special protection during flights at great speed, but they cannot afford to lose a fraction of their acuity of vision during hunting [79]. Raptors sweep the semitransparent membrane across the eye with great rapidity and at frequent intervals, without closing the other eyelids, so it does not interfere with their vision greatly while maintaining the protection of the eye [77–79]. The movement of the nictitating membrane over the surface of the cornea in Strigiformes is not swift like in other birds [77, 78] which may be caused by its thickness.

Differences in the histological structure of the eyelids were observed not only between owls and the other two orders of birds, but also between representatives of Accipitriformes and Falconiformes. In the examined species, the bundles of muscles in the upper and lower eyelid were well developed with the exception of Steppe Eagle and White-tailed Eagle. This very poor development of these muscles in the case of these two eagles may indicate that their eyelids have weaker mobility.

Moreover, it was found that the number layers of cells forming the epithelium covering the anterior and posterior surface as well as the marginal zone of both eyelids was small in many analyzed representatives of Falconiformes and Accipitriformes species. However, in Steppe Eagle and White-tailed Eagle, the number of cell layers in this part of the eyelid was much greater, similar to that observed in Eurasian Eagle-Owl and Snowy Owl. This may indicate that in larger species of birds the epithelium covering the anterior and posterior surface and the marginal zone of both eyelids has more layers. Moreover, large differences between both orders were demonstrated in the presence of clusters of melanin in such structures as the lamina propria located under the anterior epithelium, the epithelium covering the marginal zone, and in the stroma of both eyelids. Melanin was absent in the Eurasian Goshawk, Steppe Eagle and Eurasian Kestrel. A number of cell layers forming the marginal zone of the upper and lower eyelids between the analyzed representatives of Accipitriformes and Falconiformes was very diverse. Large differences in the number of cell layers forming the epithelium covering both eyelids have also been observed in various bird species described [12–14]. Such a large variation may indicate a large environmental impact on this eyelid feature, or it may be related to the variability of an individual. The size of the tarsal plate located in the lower eyelid varies between species. In species with relatively small eye sockets like Peregrine Falcon and Eurasian Kestrel, the tarsal plate was smaller than in the Steppe Eagle and White-tailed Eagle, whose tarsal plate was the largest.

In all examined birds, a relatively modest number of well-differentiated goblet cells were found on the palpebral conjunctiva. It corresponds with previous results [11], especially for aquatic birds where numerous goblet cells were observed [14]. Glycoprotein mucins present in the goblet cells are an essential component of the precorneal tear film, designed to tightly adhere and maintain the tear film on the corneal surface.

Moreover, in the upper and lower eyelids of all examined birds, distinct histological differences in the structure of the CALT were observed in the form of intraepithelial lymphocytes, subepithelial lymphocytes, lymphoid follicles, and adjacent lymphatic and blood vessels, as well as numerous scattered diffuse lymphatic cells similar in the Bilgorey Goose and the other species of birds [13, 14]. The presence of typical CALT was found in the Accipitriformes and Falconiformes, while in the examined owls it occurred in a differentiated form, i.e. either in the form of only lymphoid follicles or only diffuse lymphocytes with HEV or as typical CALT. Chodosh et al. [80], Knop et al. [81] and Sibelmann et al. [82] reported that lymphoid follicles were covered by a follicle-associated epithelium devoid of goblet cells. Histological studies of conjunctival epithelium performed on ostriches by Bayraktaroglu et al. [83] showed the occurrence of epithelium containing a non-lymphoid region with goblet cells and a lymphoid region with the absence of goblet cells. In the case of the species White-tailed Eagle, European Honey-buzzard, Eurasian Sparrowhawk, Ural Owl, Western Barn Owl, Boreal Owl, and Tawny Owl, it was shown that the lymphoid follicle was located in the area of the posterior epithelium covered with goblet cells. However, in the Boreal Owl, Eurasian Eagle-Owl, Tawny Owl, and Northern Hawk Owl the non-lymphoid region is characterized by numerous goblet cells.

In all analyzed species, the CALT was more extensive in the lower eyelid than in the upper eyelid similar to the Common Ostrich (*Struthio camelus*) [12], and the chicken (*Gallus gallus domesticus*) [27].

Histological analysis of the third eyelid in the Accipitriformes and Falconiformes showed that the marginal plait stroma has numerous clusters of melanin around the blood vessels, as well as their presence in the stroma of the third eyelid itself, with the exception of Peregrine Falcon. Such a lack of granules of melanin in this species of bird may be justified by the fact that during a very quick dive towards the prey, the moving eyelid, being transparent, ensures undisturbed visibility. Moreover, different numbers of palpebral folds and bulbar folds were observed between representatives of Accipitriformes and Facloniformes. Also, pigmentation of marginal plait

epithelium was observed in the Eurasian Sparrowhawk, Common Buzzard, Hen Harrier, Steppe Eagle, European Honey-buzzard, and Peregrine Falcon.

Conclusions

The structure of the upper, lower and third eyelids in the studied representatives from Accipitriformes, Falconiformes and Strigiformes orders turned out to be very diverse despite some common features. The third eyelid, as well as CALT, proved to have the most diverse morphological structures among the analyzed species.

Owls turned out to be a group of raptors characterized by the most outstanding differences in eyelid morphology. This group of birds is not only different from other raptors but also very internally diverse and many significant differences were observed between individual owl species. However, it is not possible to explain all observed differences only as an adaptation to a nocturnal lifestyle because many owl species have diurnal activity.

It is also impossible to assess individual diversity due to an insufficient number of species analyzed and a small number of individuals representing individual taxa. Additional research is necessary to outline the phylogenic background and understanding of the significance and evolutionary history of the observed morphological traits. However, the results obtained in the presented study provide more data for further comparative studies of eyelids in birds.

Abbreviations

CALT	conjunctiva-associated lymphoid tissue
EALT	eye-associated lymphoid tissue
FAE	follicle-associated epithelium
H&E	hematoxylin and eosin stain
HEV	high endothelial venules

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Author contributions

J.K.N. Conceptualization;, J.K.N. and K.G.H. collection and transport of material (from Wroclaw Zoological Garden); A.K.Z., K.G.H. and A.D.U. collecting birds from the field; J.K.N. and A.K.Z. investigation; A.K.Z. collecting eyelids for histological examination,; A.K.Z. and A.D.U. description of the evolutionary aspect; J.K.N., K.G.H. and K.B. histological analysis of test results; J.K.N. and K.G.H. methodology; J.K.N., A.K.Z., A.D.U., K.B. and K.G.H. visualization and writing – original draft; K.G.H. writing – review and editing. All authors reviewed the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

According to Polish and European law, studies on tissues obtained *post-mortem* do not require the approval of the Ethics Committee (Journal of Laws of the Republic of Poland Act of 15 January 2015 on the protection of animals used for scientific or educational purposes; Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes). *Post-mortem* material from the Wroclaw Zoo was obtained according to permits (No. PIW Wroc. UT-45/5/16 and No. PIW Wroc. UT- 45/6/16) issued by the District Veterinary Officer in Wroclaw (Poland). *Post-mortem* material from birds kept in private collections was obtained according to the permission (No. PU.555.15.2020) issued by the District Veterinary Officer in Wroclaw (Poland). Post-mortem material from birds found in the field was obtained according to the permission (No. WPN.6401.83.2021.MH) issued by the Regional Director for Environmental Protection in Wroclaw (Poland).

Consent to publish

Not applicable.

Competing interests

The authors declare no competing interests.

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