Pineal Gland Morphology in Relation to Age and Season in Three Canidae Species

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Abstract

Introduction The aim of the present study was to determine the morphological features of the pineal gland in three closely related Canidae species (raccoon dog, *Nyctereutes procyonoides* Gray, 1834; silver fox, *Vulpes vulpes* L., 1758; and blue fox, *Vulpes lagopus* L., 1758) of different ages during the breeding (spring) and nonbreeding (winter) periods.

Materials and Methods Histological analysis of the pineal glands of canids was performed.

Results The morphological changes in the pineal gland detected in the current study are either age-associated, including increase in the reticular fibers and vascularization in the studied species, as well as increase in the amount of the protruding septae in the blue fox, or seasonally related, including an increase in the number and size of blood vessels. The present work reported two types of pigments: lipofuscin (primarily in the silver fox) and melanin (primarily in the raccoon dog and in the blue fox). The pineal gland in the blue fox is characterized by the ability to form corpora arenacea.

Conclusions The present study provides the first insight into the morphological changes of the pineal gland in three closely related Canidae species of different ages during the breeding (spring) and nonbreeding (winter) periods, and showed some species-specific features of gland morphology. The aspects concerning the biogenesis of the calcium concretions and the factors influencing the accumulation of pigments need further investigation.

Keywords
► canidae
► compared anatomy
► corpora arenacea
► microscopic animal anatomy
► pineal gland

Introduction

The structure and ultrastructure of the mammalian pineal gland have been largely described in previous reviews.[1,2] As a major component of the photoneuroendocrine system, the pineal gland appears to be a "neuroendocrine transducer", receiving external stimuli (photoperiodic information) from the retina and the circadian suprachiasmatic nuclei (SCN) oscillator and converting this in hormonal responses (especially melatonin [MEL] production).[3,4] Therefore, the pineal gland allows mammals to respond to the daily or annual changes in the photoperiod by adaptive alterations of their physiological state. The best example of such photoperiod-dependent physiological functions in the year is the activation/inactivation of the reproductive axis, a phenomenon in which the pineal and its MEL rhythm are essential in most temperate latitude mammals,[3] including Canids.

The closely related Canids, blue fox (*Vulpes lagopus*), silver fox (*Vulpes vulpes*), and the raccoon dog (*Nyctereutes procyonoides*), are fur-bearing animals, whose physiological traits (reproduction, moulting, etc.) are specifically and reversibly regulated on a seasonal basis.[5–8] The question arises whether the morphology of the pineal gland in canids have been changing during the breeding and nonbreeding periods, as well as with advancing age. The literature contains some descriptions of pineal morphology, including age-related

changes, only in a few canids (blue fox[9]; dog[10,11]; fox[12]; and silver fox[13]). No analyses have been performed of the influence of season and age on morphology of the the pineal gland in canids.

Therefore, the aim of the present study was to determine the morphological features of the pineal gland in three closely related Canidae species (raccoon dog, silver, and blue foxes) of different ages during the breeding (March) and nonbreeding (December) periods.

Materials and Methods

The research was carried out using the equipment of the Core Facility of the Karelian Research Centre of the Russian Academy of Sciences. All of the experiments were conducted according to the EU Directive 2010/63/EU for animal experiments with the special permission of the Local Ethic Committee of the Institute of Biology.

Animals and Material Collection

The three Canidae species (raccoon dog, *N. procyonoides* Gray, 1834; silver fox, *V. vulpes* L., 1758; blue fox, *V. lagopus* L., 1758) were used (►Table 1). The animals were reared in a fur farm, "Russian sable", (Moscow region, Russia, 56°02′ N, 37°58′ E) in individual, outdoor cages. They were fed in accordance with the nutritional regime for fur-bearing animals with free access to water. The animals were sacrificed between 8 AM and 9 AM in March (breeding period; photoperiod of 12-hour light/12-hour dark) and in December (period of sexual rest; photoperiod of 07-hour light/17-hour dark), in line with the approved procedure for fur animal farms. The pineal glands were removed immediately after skinning.

Histology and Microscopic Examination

After being removed, the pineal glands were immediately fixed by immersion in 10% neutral buffered formalin at room temperature for histological preparations. The fixed glands were dehydrated in ascending series of alcohol grades, cleared in xylene, then embedded in paraffin wax and sectioned with a thickness of 5 μm in the dorsoventral or sagittal planes. The sections were then stained with Ehrlich hematoxylin and counterstained with eosin (H&E), and with Masson-Goldner for the visualization of connective tissue and silver impregnation for reticular fibers. The stained sections were then mounted in distyrene plasticizer and observed under an AxioScope.A1 light microscope (Carl Zeiss, Oberkochen, Germany). The images were made using an AxioCam MRc 5 video camera (Carl Zeiss, Oberkochen, Germany), connected to the microscope, and the image-processing system AxioVision (Carl Zeiss, Oberkochen, Germany).

Results and Discussion

Gross Morphology of the Pineal Gland in Canids – General Comments

The pineal gland develops as an evagination of the diencephalic roof. In most mammals, it forms a solid mass, extending from the region located between the habenular and posterior commissures in the caudodorsal direction. The mammalian pineal gland joins with the pineal recess of the third ventricle.[2]

The pineal gland of the studied species is classified as A or AB type according to the Vollrath classification[2] and displays large individual variability in shape and size (►Fig. 1). Generally, the pineal gland is a conical organ (up to 5–6 mm long and 3–4 mm wide) with or without invagination on the surface, and sometimes divided into 2 parts by connective tissue fibers. But, also as a result of the fact that the distal part of the gland does not increase in size during development, in the dorsoventral view, the pineal has the form of a ribbon lying in the immediate vicinity of the third ventricle. The raccoon dog often has a big conical-shaped gland with invagination on the surface; the organ of the silver fox is mostly also big and round or elongated; whereas in the blue fox two forms of pineal – ribbon-like and conical – are observed with the same frequency.

The pineal gland is surrounded by a pial capsule; the glandular parenchyma comprises the more numerous pinealocytes, cells with large nuclei of oval or round shape, less abundant glial cells, probably astrocytes, with highly heterochromatic (strongly stained) small nuclei, fibroblasts, blood vessels, reticular and collagen fibers.

Species-specific Features of the Pineal Glands in Canids

Present findings inform about the late postnatal ontogenetic and seasonal changes in the morphology of the pineal gland in three Canidae species (raccoon dog, silver, and blue foxes). However, due to the fact that the studied mammals are monoestric, it is difficult to separate the effects of age and season on the morphology of the pineal gland.

Table 1 The experimental groups and specimen numbers

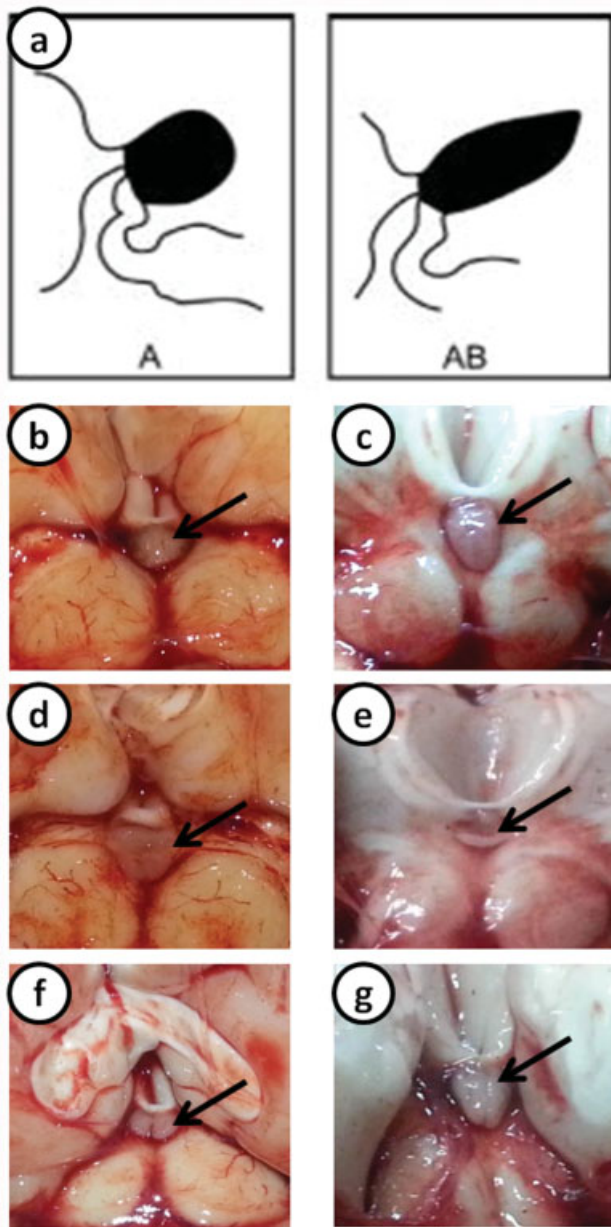| Species	Seasons and age periods			
	Winter		Spring	
	Juvenile animals (6–8 months old)	Adults in the period of sexual rest (1.5–4.5 years-old, winter adults)	Animals in the first fertile period (9–11 months old)	Adults in the reproductive period (2–5 years-old, spring adults)
Raccoon dog	$n = 4$	$n = 4$	$n = 2$	–
Silver fox	$n = 3$	$n = 4$	$n = 2$	$n = 2$
Blue fox	$n = 4$	$n = 2$	$n = 2$	$n = 2$

Fig. 1 Gross morphology of the pineal glands in Canids in situ. Caudal-dorsal view of the part of the brain after tilting of the cerebellum. The pineal gland (arrow) at the frontal edge of the colliculi is visible after slicing of the brain hemispheres. (a) Schematic representation of the A and AB forms of pineal glands according to the classification by Vollrath.[2] (b) – (g) In vivo photos, demonstrating the variability of shapes and sizes of the pineal glands: (b) oval-shaped (silver fox), (c) conical (blue fox), (d) conical with the invagination on the surface (raccoon dog), (e) ribbon-like (blue fox), (f), (g) pineal gland divided into two parts (silver and blue fox, respectively).

At the age of 6–8 months old, in the winter period, the pineal gland parenchyma in the studied Canids displays great cellularity: cells are randomly arranged, with no clear orientation (►Fig. 2). The differences in the densities of the cells and connective tissue fibers appear in the first fertile age (9–11 months old) in the spring by forming the functionally dissimilar parts: on the one side, the central and peripheral regions, and on the other side, the proximal and distal ones. In
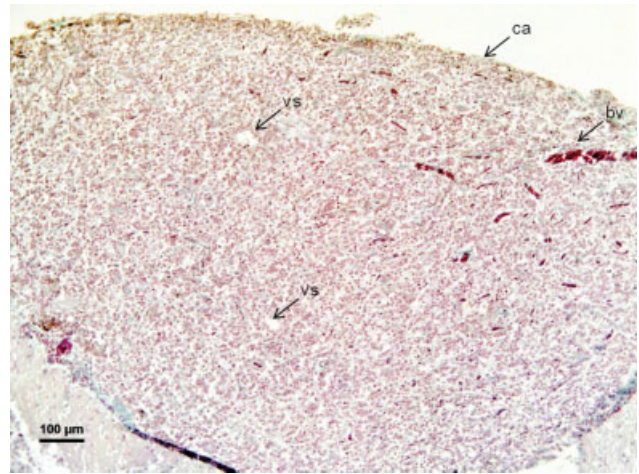

Fig. 2 Photomicrograph of a sagittal direction section of the pineal gland in a juvenile silver fox (winter). Capsule (ca), blood vessels (bv), vascular structures (vs). Masson-Goldner staining.

contrast to the peripheral area (cortex), the center (medulla) of the gland is characterized by a greater amount of cells and collagen, and by a lesser amount of reticular fibers. In the pineal of the blue fox, these differences are not so marked as in the other species studied. The distal regions of the pineal glands of the canids studied contain more reticular fibers, more pigments and more calcium concrements (if these structures appear). Our results are in accordance with previously published data that showed that the pineal cortex and medulla display differing cell densities (in sheep)[14] and that the distal and proximal portions of the pineal gland differ in structure (size of pinealocytes, amount of astrocytes, nervous fibers, and fenestrae of capillary endothelium) (in cotton rats)[15].

Light and electron microscopy has allowed the fine description of the ontogenesis of the mammalian pineal gland (in rats[16,17]; dogs[10,11]; horses[18]; sheep[14]; and in humans[19]). But these data are contradictory. Several authors noted regressive signs in morphology, such as the increase in connective tissue fibers and calcium content (in rats[16]), but others (in humans[19]) revealed that the parenchymal cells of the gland do not undergo atrophy in adult life and there does not appear to be any pattern in the development of fibrosis or of gliosis in the pineal gland.

The amount of connective tissue fibers in the studied mammals (mostly collagen ones in raccoon dog and mostly reticular fibers in foxes) is increased with age, which is confirmed by the data obtained on rats[16] and rabbits.[20] In the winter and spring, the gland of adult blue foxes has a marked loose structure due to the great amount of reticular fibers. In the winter period, marked parenchymal cell clusters separated by connective tissue trabeculae extending from the capsule are observed.

Previously, it was defined that the minimum rate of rat pineal blood flow per gram exceeds that of most endocrine organs, equals that of the neurohypophysis, and is surpassed only by that of the kidney.[21] Throughout life, the pineal gland in canids has a rich blood supply, but during the periods of reproductive activity (spring), the number or the size of blood

vessels is increased compared with the period of sexual rest (winter). These findings confirm the well-known important role of the pineal and its hormone MEL in the photoperiodic control of reproductive functions in seasonally-breeding mammals.[4] However, according to other researchers, in blue foxes, changes in the MEL level during the year were not observed;[22] in the raccoon dog, the blood plasma concentration of MEL in December was higher than in March;[23] and in the silver fox, contrarily, its level was higher in March than in December.[24]

In a 1.5-year-old raccoon dog, the pinealocytes in the central region of the gland form glandular structures with a central lumen, bordered with epithelial cells (►**Fig. 3**). Similar findings were also revealed in bovine embryos,[25] in which the appearance of these structures could be related with the act of MEL secretion. Other vascular structures are visible in the parenchyma at all of the studied ages (►**Fig. 2**).

Rare mitoses are observed in animals in the 1st year of life, and are found in adults in the winter and spring. Degenerated cells, both pinealocytes and fibroblasts (with pyknotic, karyolitic and karyorrhexic structures), are frequently observed in the pineals of the raccoon dog and of the blue fox at the 1st year of life, but their occurrence is decreased with age (►**Fig. 4**).

Similarly to other authors,[16,26–29] we have also detected marked differences in the staining intensity and in the chromatin state in the nuclei of pinealocytes. Conditionally, we divided the nuclei in three types: dark (with more uniformly distributed and dense chromatin, and a hardly discernible nucleolus), light (light staining nuclei with ≥ 1 visible nucleoli), and intermediate (has intermediate staining features and finely dispersed chromatin without prominent nucleoli) (►**Fig. 5**). Previous findings based on electron microscopic,[29] electrophysiological[30] and immunohistochemical[31] investigations of the pineal gland provide reason to suspect that the population of pinealocytes is highly heterogenic, and that MEL synthesis varies significantly among individual cells. The light and intermediate types of nuclei are more numerous than dark ones at the all studied ages in any season.
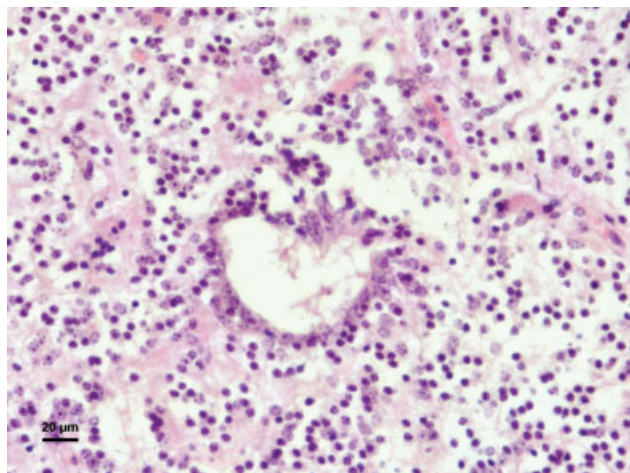

Fig. 4 Photomicrograph of a section of the pineal gland in a juvenile blue fox (winter), central area. Nuclei (purple), connective tissue fibers (blue); pyknotic nuclei (black). Masson-Goldner staining.

In several Canids (raccoon dog[23]; silver fox[24]; dog[32]) and other seasonally breeding animals (European hamster[33]) a marked seasonal variation in MEL synthesis was detected, whereas it was not observed in the blue fox.[22]

The present work reported two types of cytoplasmic pigments, which according to the morphology and staining features, most probably correspond to lipofuscin (yellowish-brown) and melanin (strong argirophyl reaction, as shown by silver impregnation). The pineal gland of the silver fox contains a small amount of yellowish-brown pigments during the 1st year of life; it increases in the adults, especially in the winter period, and is found mostly in the periphery of the proximal part of the gland (►**Fig. 6**). In contrast with the silver fox, the other studied species have very small amount of yellowish-brown pigments in their pineal glands throughout life (►**Fig. 5**).

Highly pigmented black-colored glands were observed among the raccoon dog (►**Fig. 7**) and the blue fox at the ages between 6 and 8 months old. In these cases, the pigment was melanin. Our finding is in line with the data of Capucchio

Fig. 3 Photomicrograph of a section of the pineal gland in adult raccoon dog (1.5 years old, winter), the proximal part of the gland. Round glandular structures with a central lumen, bordered with epithelial cells. H&E.
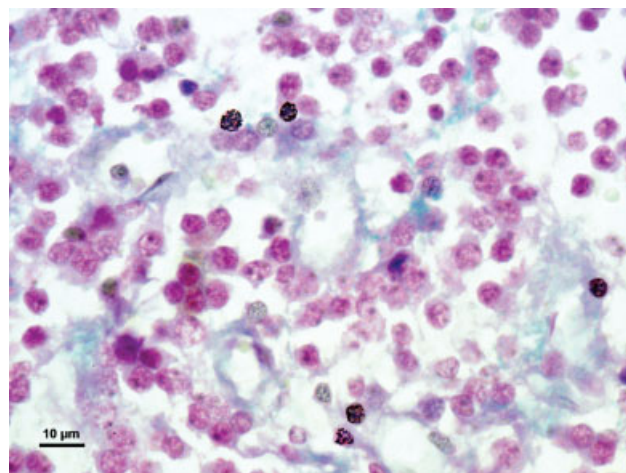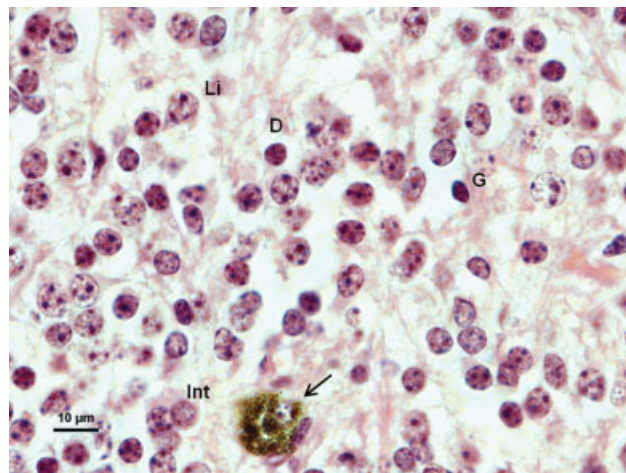

Fig. 5 Photomicrograph of a section of the pineal gland in a juvenile raccoon dog, central area. Pigment granules (arrow), nuclei of glial cells (G), "light" (Li), "intermediate" (Int) and "dark" (D) types of nuclei of pinealocytes. H&E.
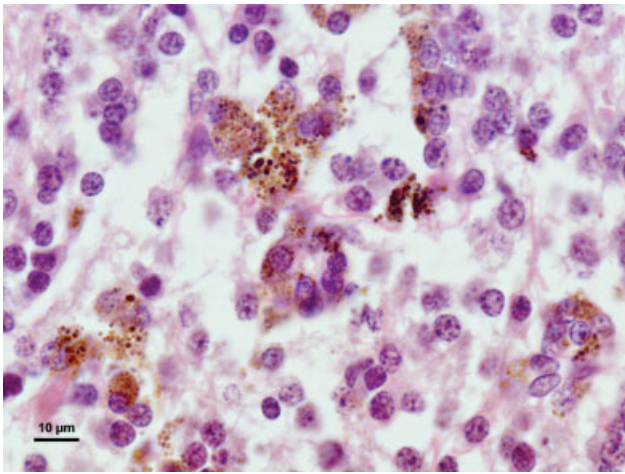
Fig. 6 Photomicrograph of a section of the pineal gland in an adult silver fox (winter), proximal part of the gland. Large accumulation of pigment granules. H&E.

et al,[18] who considered that the presence of melanosis is unrelated to the age of horses. Also, Quay[34] has not found any relationship between the amount of pineal pigment with age, gender, or the reproductive state in rodents. In the pineal glands of dogs and cats, the pigment is normally found.[35,36] However, several authors revealed that the increase in the amount of melanin in the pineal gland could be defined with increasing age (in humans[19]) or after exposure to constant darkness (in bats[37]).

Considering the fact that the mammalian pinealocytes are phylogenetically derived from the true photoreceptors, the pineal melanin could represent a phylogenetic relic whose developmental history can be traced to the pigmented epithelium of the retina.[37] Pigmented cells may constitute a special type of pinealocyte according to their ultrastructural features and the presence of pigment granules.[35] Alternatively, pineal melanin may be another structure in the mammalian pineal gland which has no apparent function. Pineal components that have no recognized function include synaptic ribbons, cilia and ciliary precursor materials, intrapineal neurons, pinealocyte intermediate filaments, and acervuli (calcium concrements, brain sand, corpora arenacea).[37]

The last structures are the most intriguing elements in the pineal gland. Besides that, the corpora arenacea could also occur in the leptomeninges, in the habenular commissure, and in the choroid plexus.[38] The pineal gland is able to form the deposits due to the high calcium content and the high phosphate turnover compared with other tissues.[38,39] The pineal brain sand has long been studied because of its association with aging,[38,40,41] MEL production,[42] or neurological disorders,[43] but the biological significance of pineal calcium concrements, however, is still unknown. The relevance of acervuli to aging is still arguable; in general views, their incidence and amount are believed to increase with age[44,45] despite several irrelevant cases.[19,46] It is also assumed that formation of concrements could increase during the reproductive period in connection with the functional load on the pineal gland;[41] therefore, the acervuli could possess a dynamic nature.[47]
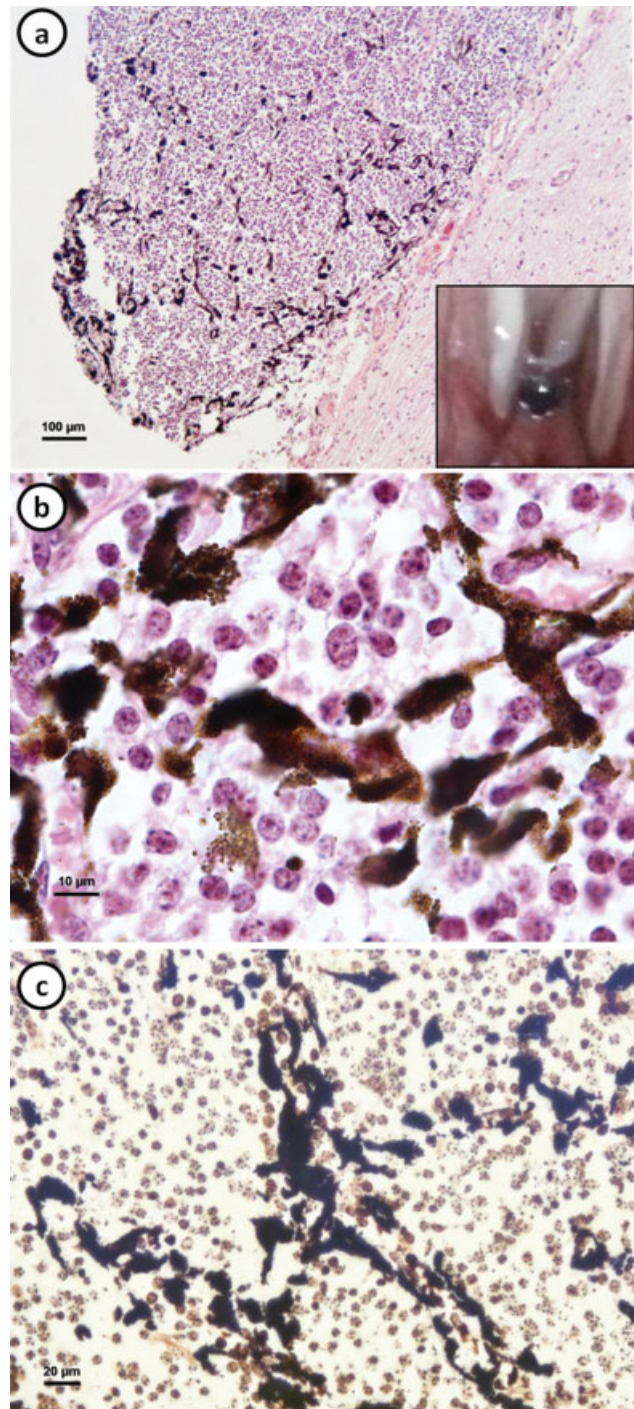

Fig. 7 Photomicrograph of the highly pigmented black-colored pineal gland in a juvenile raccoon dog (6–8 months old, winter). (a) Distal part of the gland. In the bottom right – macroscopic view of the gland. (b), (c) The magnified sectors of the distal part of the gland with abundant melanin aggregations. (a), (b) H&E, (c) Silver impregnation.

Brain sand is often found in the pineal gland of humans[48] and of many mammals (rodents[28,49]; carnivorous[9,38]). However, in our study, it was revealed that in contrast with the pineal glands of the silver fox and of the raccoon dog, the pineal gland of the blue fox appears to have the greatest ability to form calcium concretions, visible at light microscopic level, at all of the studied ages. In 1 of 4 studied

raccoon dogs aged 1.5 years old, we observed only 1 acervulus ~15 µm in diameter located in the pineal capsule, whereas in the gland of the silver fox we detected the possible site of the calcification. In the pineal gland of the blue fox, the calcified concrements were found in the parenchyma of the distal part of the gland, in the capsule surrounding it, and in the protruding septae (►**Fig. 8**).

At the light microscopic level, they are observed at the all studied ages (in 1 of 3 animals aged between 6 and 8 months old, in 2 of 2 aged between 9 and 11 months old, in 1 of 2 winter adults and in 1 of 2 spring adults), but in not all individuals. The size of the deposits is up to 40 µm. Some glands showed accumulation of numerous deposits of different sizes, whereas the others revealed only few acervuli.

Other authors revealed the differences in the structure of capsular concrements and of parenchymal ones.[38] The first show a clear concentric lamination, but the second have a globular structure. As the capsule and septae of the pineal are formed by the arachnoid and pia mater sheats of the meninges, the concrements of the capsule and septae are an example of meningeal calcification.[38] Similarly, in our study, the acervuli from the capsule had a laminated structure, but most of the concrements formed in the pineal parenchyma also had the alternative layers of light- and dark-stained rings (►**Fig. 8**). Others were strong dark-stained and third were light-stained and seemed to be the hollow structure (in sections stained with H&E) (►**Fig. 8 c, e**). However, in samples stained according to the Masson-Goldner method, the acervuli colored pink, light green, or both, and some of them had black rings, which indicated the different composition of the concrements (►**Fig. 8 b, d**). In the immediate vicinity of the acervuli, the denegerated cells, fibroblasts, and collagen fibers are often observed.

To date, there is no clear understanding of how and why calcium concretions form, but some mechanisms for their formation are suggested. Among them the impairment of calcium ion-dependent ATPase, changes in calcium channels or a constant depolarization of pinealocytes calcium pump leading to elimination of calcium out of the cell[50]; the death or degeneration of the pinealocytes, resulting in an overall
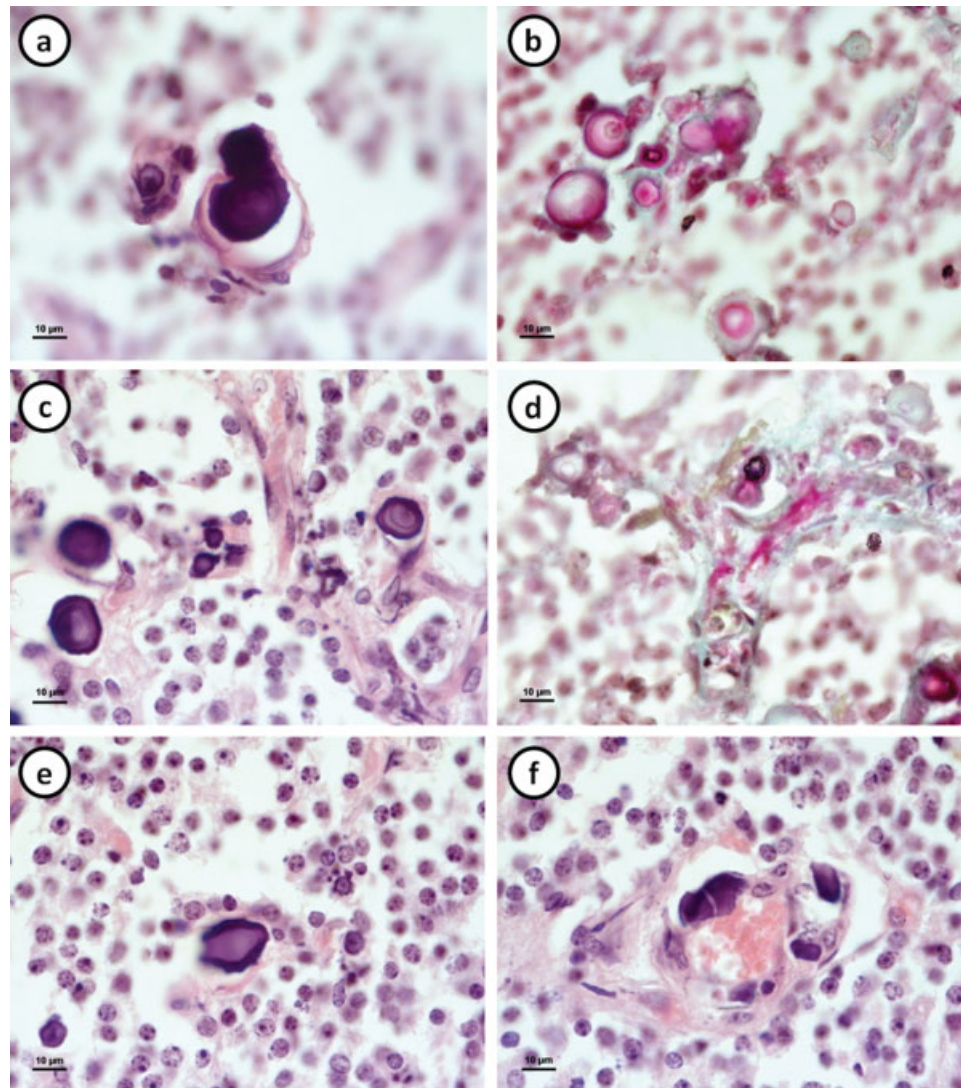

Fig. 8 Various locations and shapes of corpora arenacea in the pineal gland of adult blue fox (1.5–4.5 years old, winter). Acervuli are in parenchyma: (a) Hematoxylin and eosin. (b) Masson-Goldner staining. Acervuli are in trabeculae: (c) H&E. (d) Masson-Goldner staining. (e) Acervulus has a hollow structure. H&E. (f) Calcification of blood vessel. H&E.

decrease in the pineal gland activity[28,51] and the active participation of the tryptase mast cells in the pineal calcification process as sites where it starts.[46]

Reviewing the data available about pineal calcification, one can conclude that a multifactorial mechanism may be responsible for its formation.[38] Moreover, the nature and crystallinity of the inorganic tissue of the pineal concretions provide reason to assume that the corpora arenacea is of a physiological rather than pathological ossification type with characteristics between enamel and dentine, but with more marked analogies toward the latter.[48]

Conclusions

The present study provides the first insight into the morphological features of the pineal gland in three closely related Canidae species (raccoon dog, silver, and blue foxes) of different ages during the breeding (spring) and nonbreeding (winter) periods. The morphological changes in the pineal gland detected in the current study are either age-associated, including an increase in the reticular fibers and vascularization in the studied species, as well as an increase in the amount of the protruding septae in the blue fox, or seasonally related, including an increase in the number and size of blood vessels. The present work reported two types of pigments: lipofuscin (primarily in the silver fox) and melanin (primarily in the raccoon dog and in the blue fox). The pineal gland in the blue fox is characterized by the ability to form corpora arenacea. The aspects concerning the biogenesis of the calcium concretions and the factors influencing the accumulation of pigments need further investigation.

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