

Microanatomy of Passerine Hard-Cornified Tissues: Beak and Claw Structure of the Black-capped Chickadee (*Poecile atricapillus*)

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ABSTRACT The microanatomy of healthy beaks and claws in passerine birds has not been well described in the literature, despite the importance of these structures in avian life. Histological processing of hard-cornified tissues is notoriously challenging and only a few reports on effective techniques have been published. An emerging epizootic of beak deformities among wild birds in Alaska and the Pacific Northwest region of North America recently highlighted the need for additional baseline information about avian hard-cornified structures. In this study, we examine the beak and claw of the Black-capped Chickadee (*Poecile atricapillus*), a common North American passerine that is affected by what has been described as “avian keratin disorder.” We use light and scanning electron microscopy and high-magnification radiography to document the healthy microanatomy of these tissues and identify features of functional importance. We also describe detailed methods for histological processing of avian hard-cornified structures and discuss the utility of special stains. Results from this study will assist in future research on the functional anatomy and pathology of hard-cornified structures and will provide a necessary reference for ongoing investigations of avian keratin disorder in Black-capped Chickadees and other wild passerine species. *J. Morphol.* 273:226–240, 2012. Published 2011 Wiley Periodicals, Inc.[†]

KEY WORDS: avian; beak; Black-capped Chickadee; claw; *Poecile atricapillus*; passerine; rhamphotheca

INTRODUCTION

Beaks and other cornified epidermal tissues feature prominently in a number of common avian diseases caused by nutritional, viral, parasitic, and toxic agents (Pass and Perry, 1984; O'Toole and Raisbeck, 1997; Monroe et al., 2003; Olsen, 2003; Schmidt et al., 2003; Fletcher and Abdul-Aziz, 2008). We recently documented an epizootic of beak deformities of unknown etiology among wild bird species in Alaska and the Pacific Northwest region of North America (Handel et al., 2010; VanHemert and Handel, 2010). Our investigation of this disease, termed “avian keratin disorder,” has high-

lighted the need for additional background information on passerine hard-cornified tissues. To comprehend the pathology of avian keratin disorder, we must first understand normal microanatomy and structure, a requirement that prompted this study of the beak and claw of a passerine species.

The beak is a characteristic feature of extant birds and serves many essential and highly adapted functions. Ranging from the compact, powerful mandibles of seed specialists to the delicate appendages of nectar-feeders, beak morphologies accommodate a diversity of life history strategies. Despite remarkable variability in shape, size, and function, basic features of gross anatomy are similar among species. The underlying premaxillary and mandibular bones form the basic shape of the beak, which is modified by local thickenings of the epidermis (Stettenheim, 2000). The epidermis is made up of tightly packed keratinocytes that migrate outward as they mature, transitioning from an actively growing germinative layer to a fully cornified layer. Laminae of cornified cells (corneocytes) form the rhamphotheca, a horny sheath that comprises the outer surface of the beak. The rhamphotheca is continually replaced via growth and maturation of the epider-

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mis and subsequent wear from pecking and feeding (Lüdicke, 1933; Lucas and Stettenheim, 1972; Cooper and Harrison, 1994). This external layer of hard-cornified tissue provides a strong, durable structure for feeding, preening, breeding, and defense (Stettenheim, 2000). Classic studies on beak morphology and natural selection, in which Darwin's finches have featured prominently, demonstrated that even subtle differences in beak size and shape can result in profound consequences for survival and fitness (Bowman, 1961; Boag and Grant, 1981; review in Abzhanov, 2010). Current research continues to explore the ways in which resource availability and use influence beak morphology (Badyaev et al., 2008; Badyaev, 2010; Soons et al., 2010) and to highlight the functional and evolutionary importance of specific anatomical features within the beak (review in Stettenheim, 2000; Clayton et al., 2005).

The claws also aid in many essential activities of birds, including food procurement and handling, perching and locomotion, and nest construction. The basic structure of the claw includes a bony terminal phalanx covered by a thin layer of connective tissue, a basement membrane, and the actively growing layer of the epidermis, which underlies dorsal and ventral plates of hard-cornified epidermis (Lucas and Stettenheim, 1972; Stettenheim, 2000; Homberger et al., 2009). Claws have been the subject of extensive study for their proposed role in evolution of flight (Yalden, 1985; Feduccia, 1993; Glen and Bennett, 2007). Like beaks, claws also demonstrate a wide variety of morphological adaptations in extant species (Partridge, 1976; Landmann and Winding, 1995; Moyer and Clayton, 2004).

Interspecific differences in beak and claw structures that are apparent at a macroscopic level have received the greatest attention from evolutionary biologists and ecologists, but similarly diverse adaptive features also occur at a microscopic level. For example, both beaks and claws contain mechanoreceptors that perceive sensory information such as vibrations, pressure, and temperature, all of which confer information about foraging substrates and potential prey (Heppleston, 1970; Lucas and Stettenheim, 1972; Gottschaldt, 1985; Gentle and Breward, 1986). These structures, which may be densely packed in bony pits near the tip of the beak in what has been described as a bill tip organ, have attracted considerable research interest for wading birds that feed by probing in soft substrates (e.g., Zelená et al., 1997; Piersma et al., 1998; Cunningham et al., 2007, 2010) and other species that use the beak extensively for food manipulation (Ziswiler and Trnka, 1972; Krulis, 1978 and references therein). Magnetoreceptors located in the beak assist in orientation and migration in a variety of species (Fleissner et al., 2007; Falkenberg et al., 2010).

The beak may also serve a function in thermoregulation, as demonstrated in Toco Toucans (*Ramphastos toco*), which exhibit vascular mechanisms for controlled heat exchange (Tattersall et al., 2009). These examples of specialized features in beak and claw microanatomy highlight their functional significance and demonstrate taxon-specific adaptations.

Despite the importance of beaks and claws in avian life, few published sources address normal structure or provide basic histological descriptions. Of these, many describe developing birds, rather than adults, and most focus on poultry or other species of commercial interest (Kingsbury et al., 1953; Yasui and Hayashi, 1967; Lucas and Stettenheim, 1972; Gentle and Breward, 1986; Gentle et al., 1995; Kuenzel, 2007). In a seminal publication on beak growth and anatomy, Lüdicke (1933) examined the histological structure of the beak in a taxonomically diverse selection of birds, which was followed by Menzel and Lüdicke's (1974) investigation of several psittacine species. Other research on free-ranging species, including shorebirds (Scolopaci and families within Charadrii), waterfowl (Anatidae), and finches (Fringillidae) has typically focused on the identification of specific sensory structures and their importance in detecting, procuring, and manipulating food items (Krogis, 1931; Ziswiler and Trnka, 1972; Gottschaldt, 1974; Berkhoudt, 1976; Krulis, 1978; Piersma et al., 1998; Cunningham et al., 2007, 2010). Several recent publications have also described notably unusual or exotic beaks and accessories (Homberger, 2001; Seki et al., 2005, 2006, 2010; Chen et al., 2008; Tattersall et al., 2009). However, additional study of beaks and claws in passerine species using modern imaging techniques is necessary for future research on functional anatomy and pathology. In addition, few published guidelines exist for effective histological processing of avian hard-cornified structures. Preparing slides from cornified tissues is notoriously difficult due to their hardness and the variable density of their composition (Lucas and Stettenheim, 1972; Pass, 1989; Homberger et al., 2009), making investigation of avian keratin disorder and other research on pathology of beaks and claws challenging.

Here, we describe the histology and microanatomy of beaks and claws from a common North American passerine, the Black-capped Chickadee (*Poecile atricapillus*). This species occurs in Alaska, Canada, and the northern two-thirds of the contiguous United States and forages for insects, seeds, berries, and animal fats throughout the year (Foote et al., 2010). In addition to serving as a model for beak and claw microanatomy of a passerine species, Black-capped Chickadees in Alaska display a high prevalence of the recently described avian keratin disorder and are therefore priority subjects for upcoming disease investigation (Handel et al.,

2010). In this study, we use light and scanning electron microscopy (SEM) and high-magnification radiography to document normal beak and claw microanatomy, identify features of functional significance, and discuss methods for preparing histology slides from hard-cornified tissues.

MATERIALS AND METHODS

Sample Collection

We collected a total of 18 adult Black-capped Chickadees (8 males, 10 females) in the spring and fall of 2008 from various locations in south-central and interior Alaska using funnel traps and mist nets (Handel et al., 2010). We used a subset of birds that were obtained in the fall ($n = 13$) in a separate study (C. Van Hemert, unpublished data) and then opportunistically sampled these at its termination in April 2009. We euthanized birds with isoflurane using the open drop method and kept bodies cool ($\sim 4^{\circ}\text{C}$) until necropsy, which we completed within several hours of death. We conducted our work under guidance of the University of Alaska Fairbanks (UAF) and the USGS Alaska Science Center Institutional Animal Use and Care committees (Assurance #07-49, 08-57) and complied with all pertinent legal requirements.

Laboratory Methods

After necropsy, we fixed whole specimens in 10% neutral buffered formalin for 72 h and then transferred them to 70% isopropanol for long-term storage. We used high-magnification radiography (Dage XD7500NT; Nordson Corporation; Westlake, Ohio) to examine the underlying bone structure of the beak from a subset of specimens ($n = 14$). We collected images of the head and beak at $20\times$ magnification (40 kV, 2.8 W) using frame averaging ($n = 256$) with Dage XiDAT software (Nordson Corporation; Westlake, Ohio).

To reveal three-dimensional and surface structure of the beak, we examined tissues from four specimens with an ISI-SR-50 SEM. After formalin fixation, we dehydrated samples in a graded series of ethanol, vacuum dried (Tousimis-Samdri 790; Tousimis; Rockville, Maryland) them, mounted them on aluminum stubs with current-conducting tape, and sputter-coated them with gold-palladium. We viewed specimens at $100\text{--}1,000\times$ magnification using 10 or 15 kV voltage and collected images with XRD software (GBC Scientific Equipment Pty Ltd.; Braeside, Australia).

For histological processing, we subsequently treated beaks and claws with a formalin-based formic acid (8.8%) decalcification solution (Cal-Rite, Thermo Scientific; Waltham, Massachusetts) for 72 h. After decalcification, we trimmed beak and claw tissues for sectioning along either the mid-sagittal or transverse plane. For transverse sections, we cut the upper and lower beaks at four reference locations: immediately proximal to, and 1, 3, and 5 mm from the distal edge of the external nares. We removed the rear claw (hallux) of the right foot above the terminal pad and processed it intact for sectioning along the axial plane or cut it into four pieces of approximately equal length, starting at the center of the terminal pad, for transverse sections. After trimming, we placed all tissues in cassettes and stored them in 70% isopropanol for up to 60 days before processing. Using an automated tissue processor (Shandon Citadel 2000; GMI Inc.; Ramsey, Minnesota), we dehydrated samples in a graded series of isopropanol, cleared them in toluene, and then infiltrated them with paraffin. We subsequently placed tissues in a vacuum for 20 min and then embedded them in paraffin blocks (Reichert-Jung Model 8040 Tissue Embedding Center with vacuum; Reichert Technologies; Buffalo, New York). To minimize damage to the tissue, we embedded the entire claw, thus allowing serial sectioning with the microtome to obtain a median section through the digit. We hand-trimmed the tissue along the mid-sagittal plane prior to embedding for all other samples.

We softened the hard-cornified tissues by soaking the cut surfaces of paraffin blocks in commercially available hair-removal product NairTM (Church & Dwight Co. Inc.; Princeton, New Jersey) for ~ 5 min, followed by cooling for 5–10 min on an ice bath prior to sectioning. We cut sections at $5\ \mu\text{m}$ with an American Optical 820 Spencer microtome (American Optical; Buffalo, New York), floated them in a heated ($18\text{--}30^{\circ}\text{C}$) water bath, and mounted them onto charged glass slides (VWR Superfrost Plus Micro Slide; VWR International, LLC; Radnor, Pennsylvania). We repeated the NairTM immersion and cooling steps every 4–5 sections or as necessary to facilitate cutting. We dried slides on a heat tray overnight and then stained them with hematoxylin and eosin (H&E) using standard procedures (Luna, 1968). We applied special stains to reveal structures of interest in duplicate slides from a subset of tissues. We used Masson's trichrome (American Master Tech Scientific, Inc.; Lodi, California) to demonstrate keratin, collagen, muscle, and bone (Carson, 1997). Periodic acid Schiff (PAS; American Master Tech Scientific, Inc.; Lodi, California) binds to glycogen and mucin and is useful for detection of respiratory epithelia and basement membranes (Carson, 1997). Phosphotungstic acid hematoxylin (PTAH; American Master Tech Scientific, Inc.; Lodi, California) highlights fibrin, collagen, elastin, muscle, and bone. PTAH also reveals nerve fibers and can aid in detection of sensory structures (Bancroft and Gamble, 2008).

We viewed slides with a Leica DM4500 microscope (Leica Microsystems GmbH; Wetzlar, Germany), collected images with a Leica DFC420 C camera and Leica Application Suite software (Leica Microsystems GmbH; Wetzlar, Germany), and used Adobe Photoshop (Adobe Systems Incorporated; San Jose, California) and ImageJ (National Institutes of Health; Bethesda, Maryland) software for all image processing. For descriptions, we follow the well-defined terminology used by Lucas and Stettenheim (1972) who identified two primary strata in the beak epidermis: the superficial cornified layer (stratum corneum) and the underlying germinative layer (stratum germinativum). The stratum germinativum can be further divided, from innermost to outermost, into the basal (stratum basale), intermediate (stratum intermedium), and transitional (stratum transitivum) layers. At the tomial edges of the beak, transitional cells protrude into the cornified layer, a feature which is described as a lateral column. A second column of transitional cells, termed the medial column, may also be apparent and typically originates at a point dorsal, for the upper beak, and ventral, for the lower beak, of the lateral column.

RESULTS

Beak Microanatomy

Skeletal components of the beak. The premaxillary and mandibular bones of the Black-capped Chickadee beak extend through most of the length of the rhamphotheca and are covered by dermal and epidermal layers of varying thickness (Figs. 1–3). The scaffolding of trabecular bone within the beak surrounds cavernous spaces containing marrow, demonstrated by cellular outlines of adipocytes or gaps devoid of cellular material where lipid was removed during processing (Figs. 2 and 3). At the tip of the beak, these bones are replaced by wide dermal layers and a thickened epidermis (Figs. 1, 2, and 4).

Respiratory and olfactory structures of the beak. The chickadee beak contains a variety of specialized respiratory and olfactory structures, particularly near its base. In the upper beak, hyaline cartilage of the nasal passages consists of large, basophilic, PAS-positive cells (Fig. 5). Eosinophilic connective tissue, including elastin and

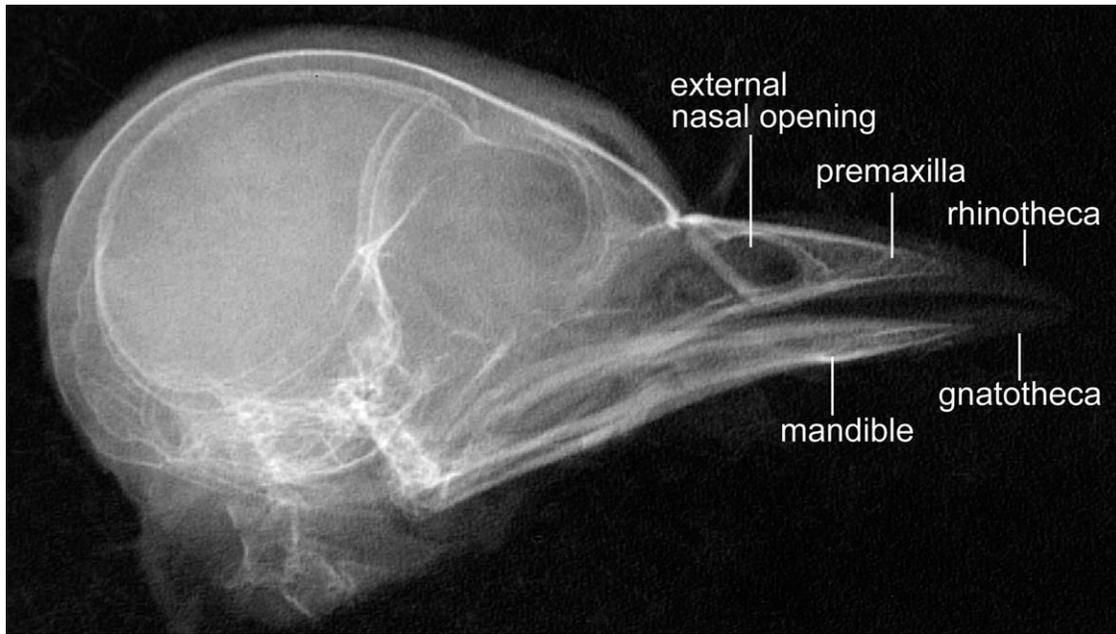


Fig. 1. High-magnification radiograph showing underlying bone structure of the beak in relation to external margins of the hard-cornified epidermis of the rhamphotheca (rhinotheca, gnathotheca) in Black-capped Chickadee.

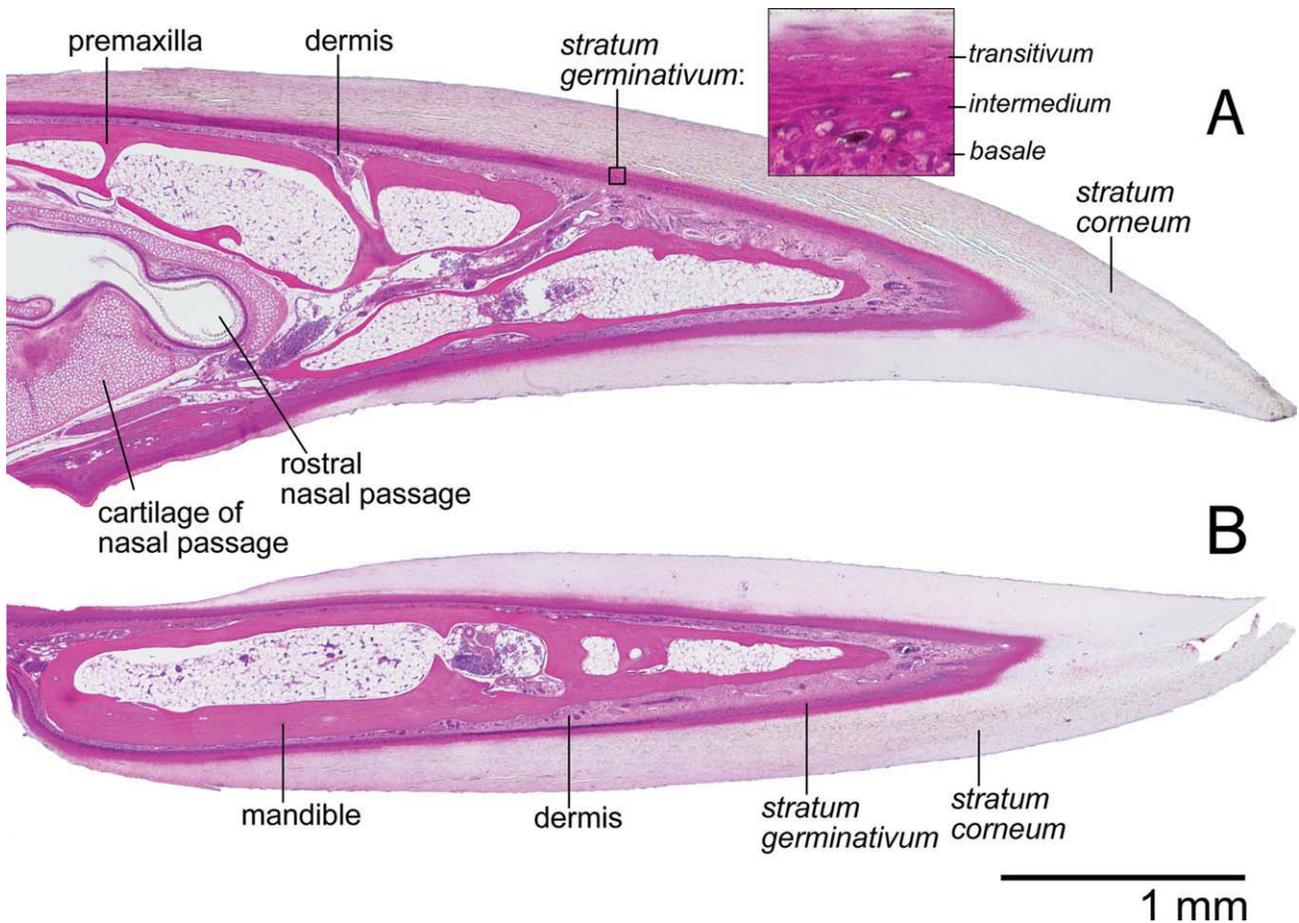


Fig. 2. Mid-sagittal section of (A) upper and (B) lower beak of Black-capped Chickadee showing underlying bone core (premaxilla, mandible), dermis, epidermis (stratum germinativum, stratum corneum), and nasal passages. Hematoxylin and eosin.

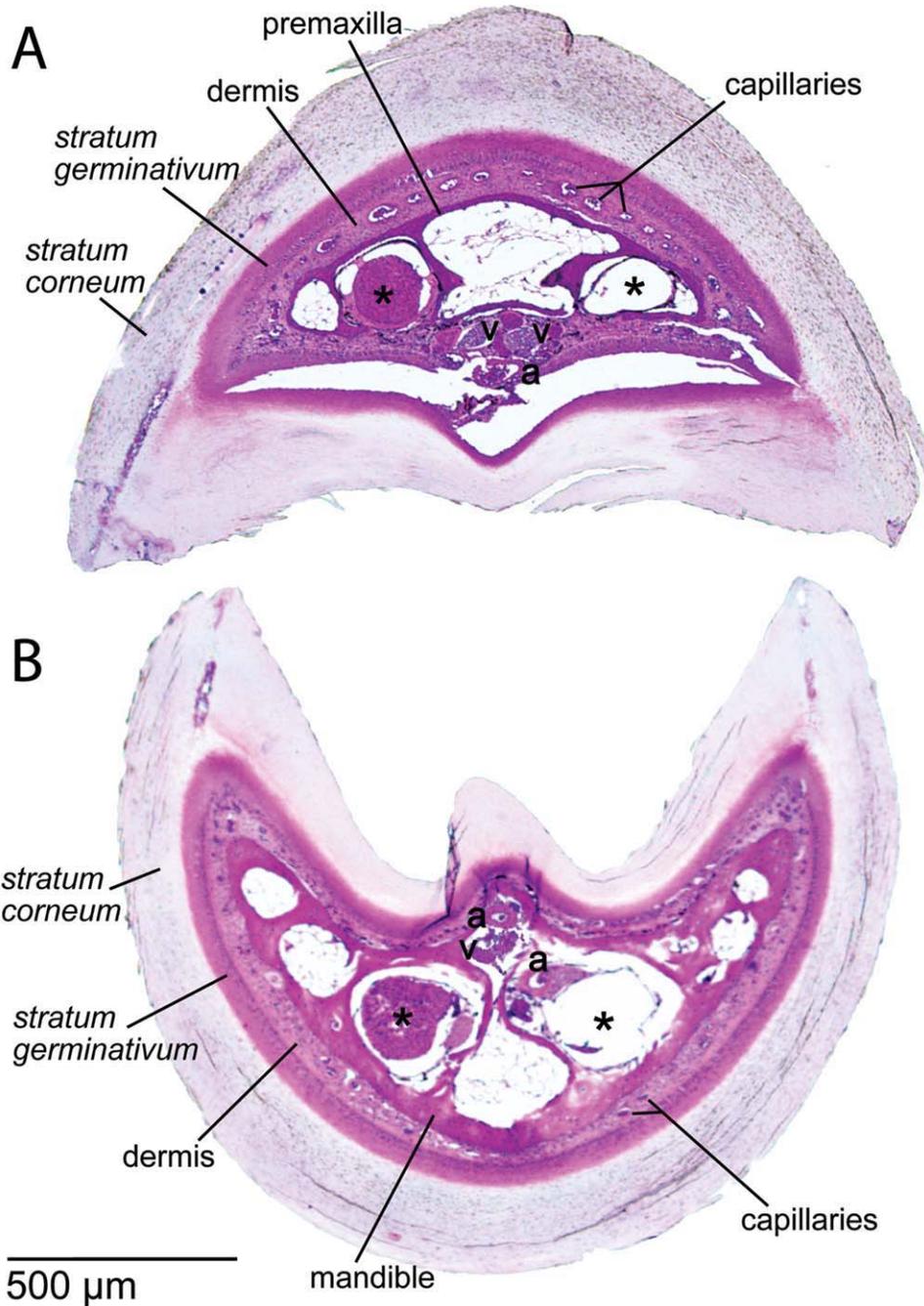


Fig. 3. Transverse sections 1-mm distal to external nares of upper (A) and lower (B) beak of Black-capped Chickadee. Note trabecular bone core and thick dermal and epidermal layers. Channels within trabecular bone house large blood vessels (asterisks) adjacent to bundled arteries (a) and veins (v). Hematoxylin and eosin.

collagen, surrounds the cartilage, providing additional support to the nasal passages (Fig. 5). The epithelial lining of the nasal passages varies throughout different regions of the beak. The rostral nasal chamber is characterized by stratified squamous epithelium (Fig. 5D), which is replaced by respiratory epithelium (Fig. 5B) in the middle chamber and infraorbital sinus and olfactory epi-

thelium in the caudal chamber. Pseudostratified, ciliated columnar respiratory epithelium with abundant mucous glands is evident in the middle nasal chamber and sinuses (Fig. 5B). Olfactory epithelium of the caudal nasal chamber is composed of a single layer of cuboidal cells (Fig. 5C). Salivary glands are visible on the buccal sides of both the upper and the lower beaks (Fig. 5C).

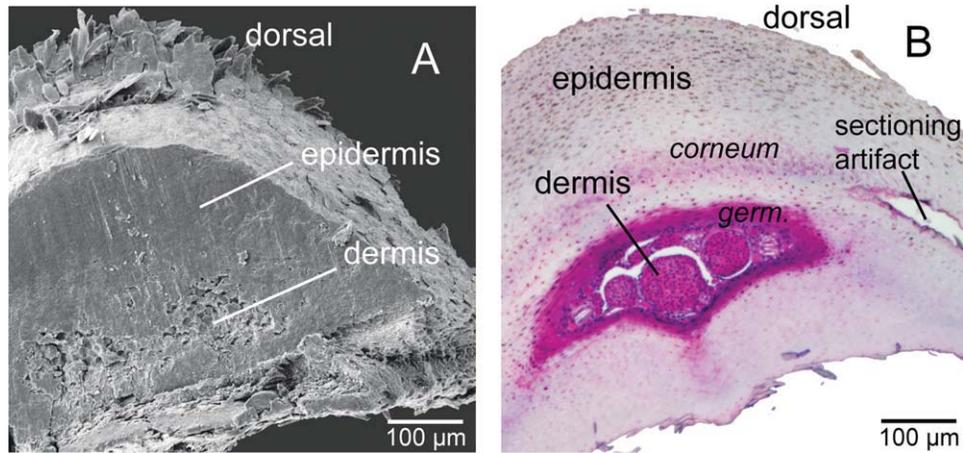


Fig. 4. Transverse sections of the upper beak of Black-capped Chickadee near its tip. Scanning electron (A) and light (B, hematoxylin and eosin) microscopy demonstrate lack of bone core and thickened dermis and epidermis, including cornified (corneum) and germinative (germ.) layers, at this distal location.

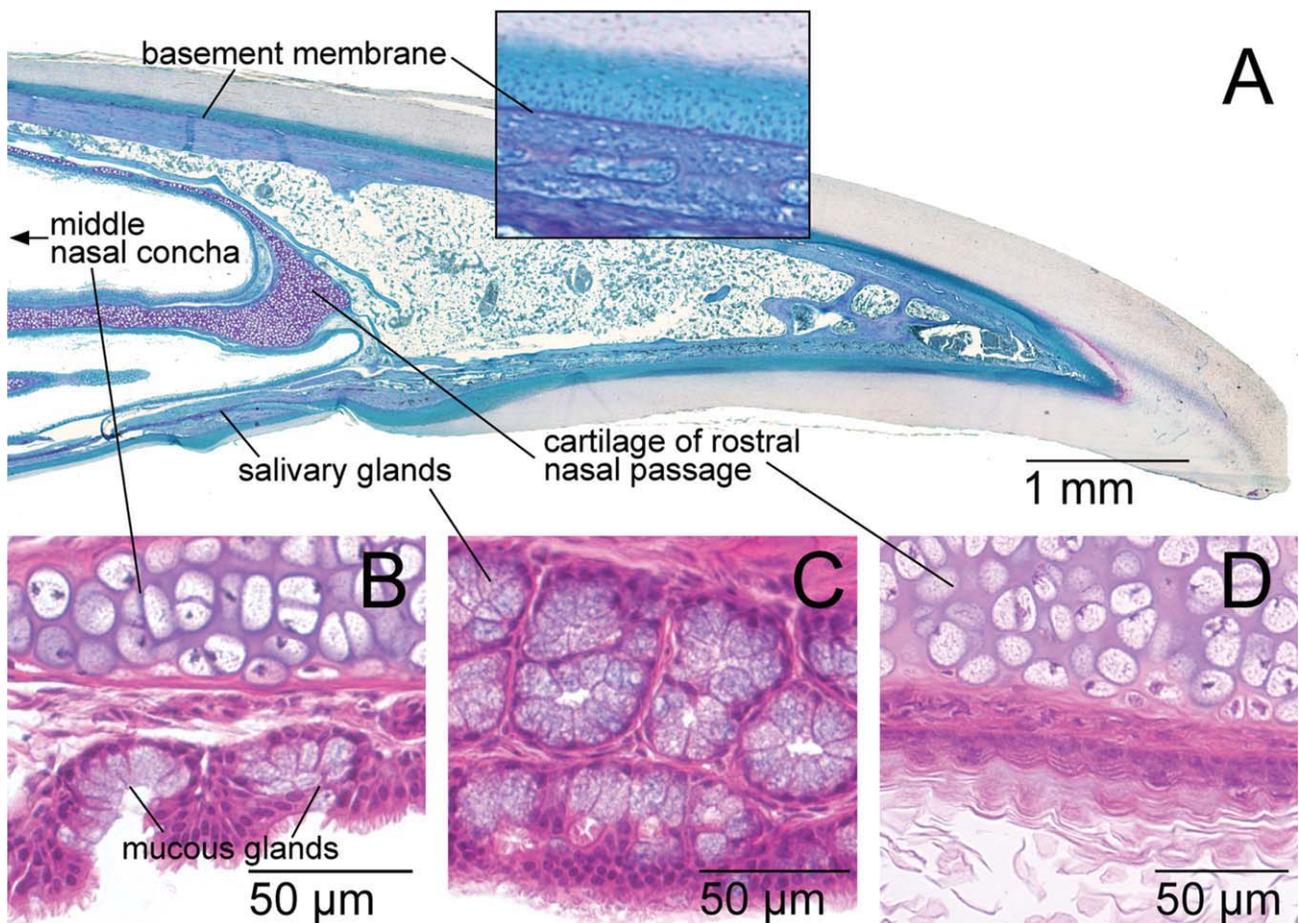


Fig. 5. Examples of specialized structures in upper beak of Black-capped Chickadee: (A) PAS-positive basement membrane, salivary glands, and cartilage of rostral nasal passages; (B) hyaline cartilage, pseudo-stratified, ciliated respiratory epithelium, and mucous glands of middle nasal concha; (C) salivary glands and cuboidal olfactory epithelium in lower beak; (D) hyaline cartilage and stratified squamous epithelium of rostral nasal passage. PAS (A), hematoxylin and eosin (B–D).

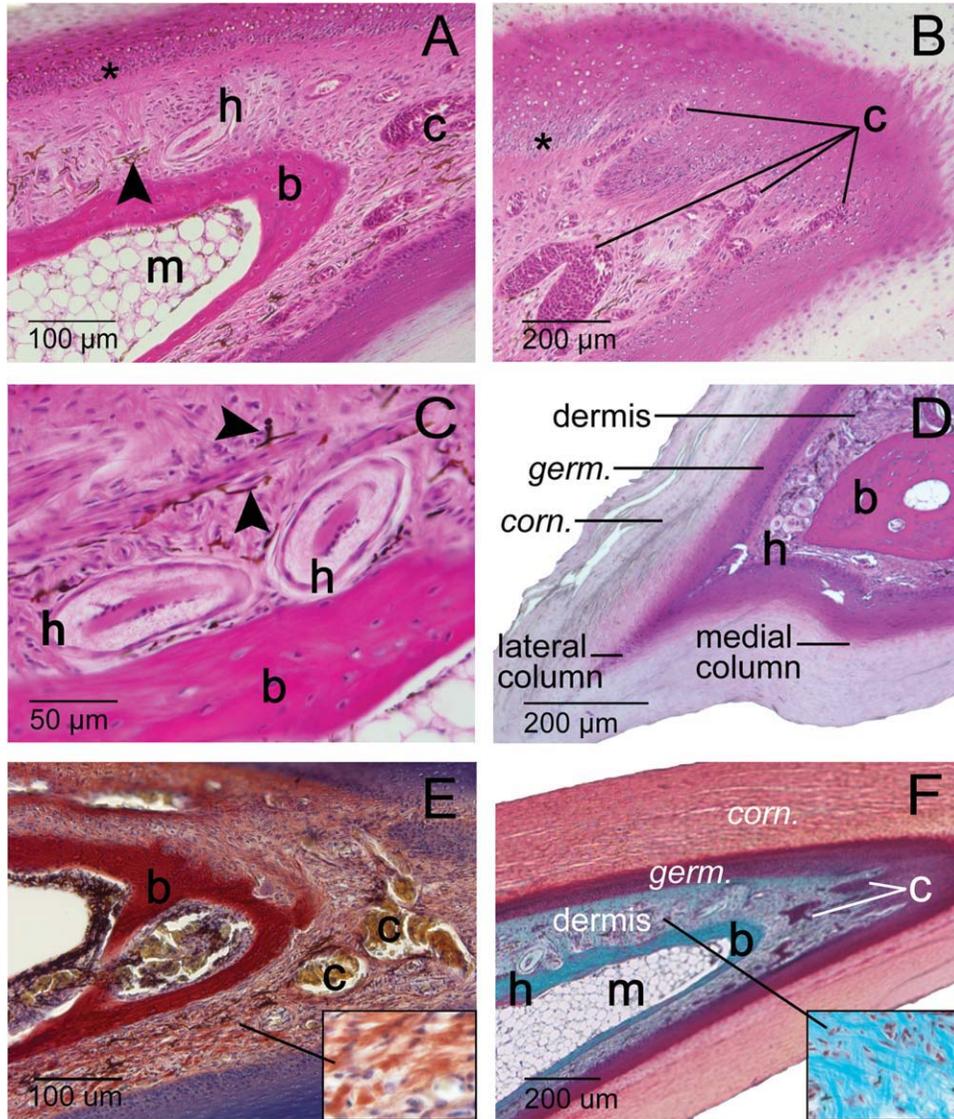


Fig. 6. Mid-sagittal sections of upper (A,E,F) and lower (B,C) beak and transverse section of lower beak (D) of Black-capped Chickadee showing bone (b), dermis, cornified (corn.) and germinative (germ.) layers of the epidermis, and lateral and medial columns. Adipocytes are visible within the marrow (m) of the premaxilla. The dermis contains Herbst corpuscles (h), melanin pigment (arrowheads), abundant capillaries and small venules (c), and collagen and elastin fibers, which are visible as dark reddish-brown fibers in (E, inset) and blue fibers (collagen only) in (F, inset). Basal cells of the epidermis (asterisks) become distally inclined near the tip of the beak, shown in (B). Hematoxylin and eosin (A–D), PTAH (E), Masson's trichrome (F).

Dermal connective tissue of the beak. The dermis of the beak is a highly vascularized layer that is proportionally thickest near the tip and contains connective tissue, blood vessels, and nerves. Collagen fibers are most densely packed along the outer margin adjacent to the epidermis, but no actual division of layers is apparent. Although the cells adjacent to the bone surface form a slightly darker band, we could not clearly distinguish a periosteum from the dense dermal tissue in either longitudinal or transverse sections.

Capillaries and small blood vessels are abundant in the dermis (Figs. 2, 3, and 6). A bundle of what

may be branches of the trigeminal and facial nerves, veins, and arteries occurs just ventral of two prominent cavities that house large vessels within the premaxilla (Fig. 3). A similar mirrored arrangement of vessels and nerves is present in the lower beak, with a reversed dorsoventral orientation relative to the mandibular bone (Fig. 3). Dermal papillae occur along the circumferential margin of the dermis underlying the soft skin at the base of the upper and lower beaks but these terminate prior to the transition to the hard-cornified epidermis of the rhamphotheca. Multiple capillaries from the tip of the dermal papillae

press against the epidermis at the tomial edges and beak tips (Fig. 6B,F), resulting in an uneven margin between these two layers of the integument.

Herbst corpuscles occur throughout the dermis and are often present immediately adjacent to the premaxillary and mandibular bones, with clusters along the tomial and lateral edges (Fig. 6). In transverse sections ($n = 5$) taken 1 mm distal to the external nares, the number of Herbst corpuscles in the dermis varied from 5 to 10 total with clusters of 1–4 near the tomial edges in the upper beak and from 4 to 6 total with clusters of 1–3 near the tomial edges in the lower beak. We also occasionally noted Grandry corpuscles, often located near the larger Herbst corpuscles, though these were more difficult to detect.

The dark beaks of chickadees contain abundant melanocytes in the dermal layer. These exhibit characteristic dendritic shapes (Lucas and Stettenheim, 1972), sometimes concentrated in dense, ribbon-like accumulations of dark brown pigment and appear in nearly all regions of the dermis (Fig. 6C).

Epidermal components of the beak. A thin, PAS-positive basement membrane separates the epidermis from the dermis (Fig. 5A, inset). This membrane begins at the base of the upper and lower beaks and is generally continuous along the epidermal margin, becoming faint and indistinct near the dermal tip. Staining affinity of this feature is also stronger on the superficial aspects of the beak and weakens or disappears on the buccal aspects, particularly near the base of the beak.

The relative thickness of the stratum germinativum is fairly consistent along the length of the upper and lower beaks (Fig. 2). Within the germinative layer, the stratum basale comprises a single layer of basal cells, which are strongly basophilic and vary from columnar near the tip to more cuboidal near the base (Figs. 2 and 7). At the tips of the upper and lower beaks, basal cells incline distally and nuclei are flattened into crescent shapes (Fig. 6B). Early mitotic figures, with characteristic condensation of heterochromatin and breakdown of the nuclear membrane, occur infrequently in this layer. The stratum intermedium is several cells thick and slightly less basophilic than the stratum basale (Figs. 2 and 7). Nuclei within these intermediate cells gradually lose definition and nuclear material begins to appear granular. In the stratum transitivum, the uppermost region of the germinative layer, cells assume a polyhedral shape with strongly eosinophilic intercellular laminae (Figs. 2 and 7). Basophilic nuclear material in this layer appears diffuse and irregular within pale, stippled cytoplasm. Transitional cells merge gradually into the stratum corneum but exhibit distinctly eosinophilic staining characteristics that contrast with the unstained appearance of the stratum corneum with H&E staining.

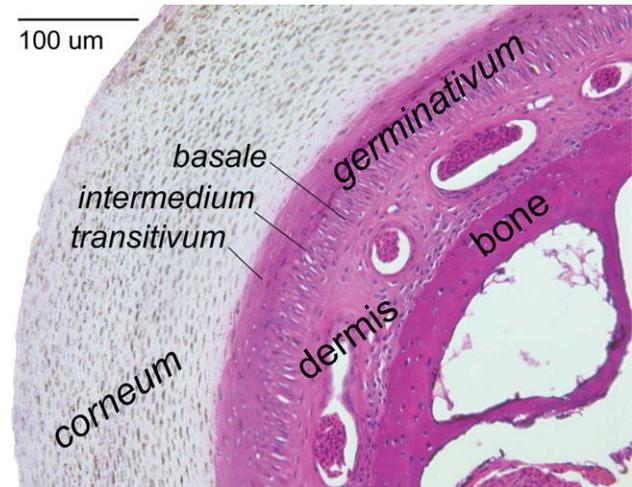


Fig. 7. Transverse section of upper beak of Black-capped Chickadee showing bone, dermis, and cornified (corneum) and germinative (germinativum) layers of the epidermis. Note dense melanin granules in cornified layer. Hematoxylin and eosin.

Cells of the stratum corneum are squamous, fully denuded, and form sheets of hard horn (Figs. 2 and 7). This layer is widest at the tomial edges of the rhinotheca and gnathotheca (the rhamphothecae of the upper and lower beaks, respectively) and also increases distally, with proportionally thicker horn layers toward the tips, especially on the superficial surfaces of the upper and lower beaks (Fig. 2). At the base of the beak, the hard-cornified layers of the rhinotheca and gnathotheca are replaced by the soft-cornified skin in the forehead and interramal regions.

The three-dimensional appearance of the flattened, longitudinally elongated polyhedral corneocytes on the surface of the rhamphotheca is shown with SEM (Fig. 8A). The superficial layers of corneocytes on the tomial surfaces and the tip of the beak appear less uniform, with many poorly adhered cells that show signs of abrasion (Fig. 8B). Sheets of mature corneocytes appear to conform generally to the shape of the beak (Fig. 8C) but exhibit possible fissures at the abrupt corners of the tomial edges (Fig. 8D).

Light microscopy also demonstrated cellular differences at the tomial edges of the beak. In transverse sections, we observed lateral and medial columns (Fig. 6D), which decreased in prominence toward the tip of the upper and lower beaks. In mid-sagittal sections, we only occasionally observed evidence of these columns, appearing as a diffuse stream of eosinophilic cells projecting a short distance into the stratum corneum at the apex of the transitional layer (Fig. 9A). When we viewed the same slides under polarized light with a lambda filter, however, a longitudinal continuation of the columns through the entire cornified layer of the rhinotheca and gnathotheca was evident (Fig. 9B). Near the tomial edges of the beak,

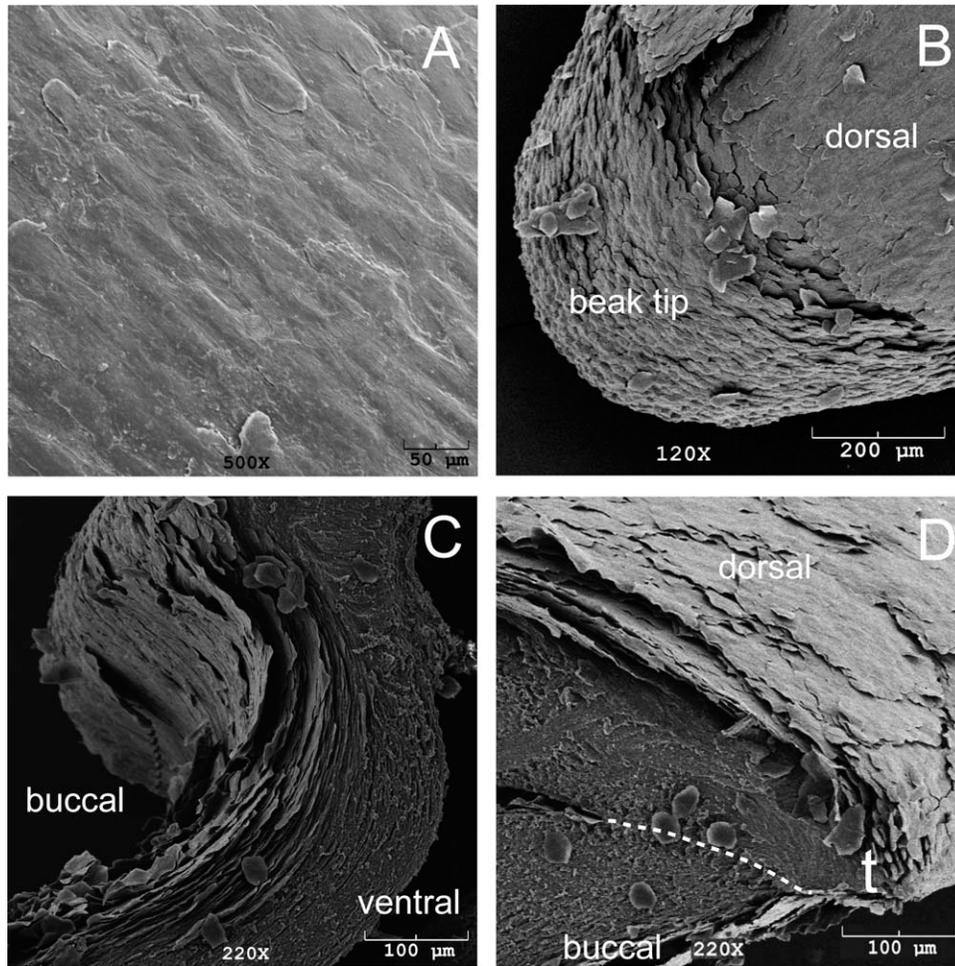


Fig. 8. Scanning electron micrographs of Black-capped Chickadee beak: (A) flattened, longitudinally elongated corneocytes on dorsal surface of upper beak; (B) dorsal view of tip of upper beak showing reduced adhesion of corneocytes at the beak tip; (C) transverse section of lower beak showing cornified laminae on buccal surface; (D) transverse section of lower beak showing right tomial edge (t) where inner (buccal) and outer (dorsal) horn layers converge with possible fissure (dashed line).

the stratum basale also appears more disorganized, with cells aligned at a variety of angles where the epidermal surface makes a sharp bend.

We observed melanocytes throughout the beak epidermis, but they were generally smaller and more compact than those found in the dermis. Pigmentation visible in epidermal layers results primarily from melanin granules that concentrate around the nuclei as they migrate outward with maturing keratinocytes (Figs. 2 and 7). Most melanin granules in the epidermis are located in superficial or tomial aspects of the upper and lower beaks and little evidence of this pigment is found within the cornified layers inside of the mouth.

Claw Microanatomy

The terminal phalanx underlies the claw and, like the bones of the beak, comprises trabecular bone with large marrow spaces (Fig. 10). The fat

tissue underlying the terminal pad and reticulate scales and scutes cushion and protect at the base of the claw (Fig. 10). The dermal layer is slightly thicker on the dorsal aspect and contains many capillaries and a dense aggregation of collagen, fibrin, and elastin. Near the tip of the claw, dermal papillae are interdigitated with the epidermis, resulting in an irregular margin between the two layers, particularly on the ventral aspect (Fig. 10). Herbst corpuscles are generally smaller and less abundant in the claw than in the beak, and we only detected them in transverse sections. Similar to the patterns we noted in the beak, dendritic melanocytes occur throughout the dermis, particularly near the base of the claw, and melanin granules appear primarily in the epidermis of the dorsal plate.

The dorsal and ventral plates of the epidermis of the claw exhibit distinct character and staining affinities (Fig. 10A). The basal cells of the stratum germinativum are cuboidal or columnar and dis-

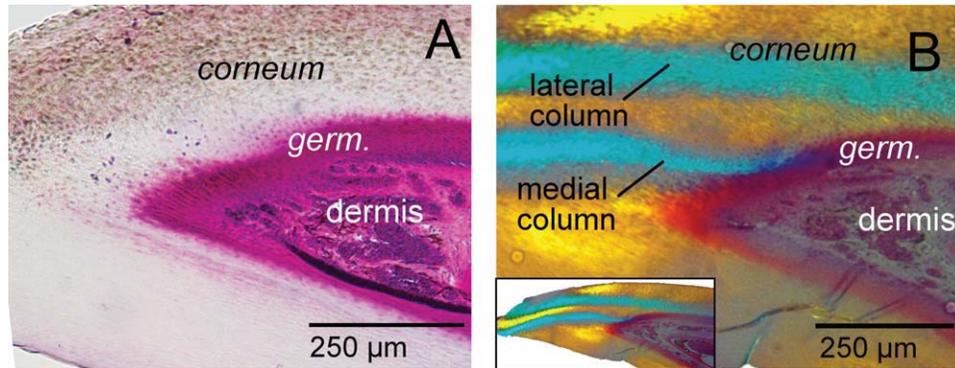


Fig. 9. Mid-sagittal section of upper beak of Black-capped Chickadee under normal light (A) and polarized light with a lambda filter (B). As shown here, lateral and medial transitional columns (blue in [B]) occur throughout the cornified layer of the beak (inset) but are detectable only with polarized light. These columns may represent a structurally significant junction between the inner and outer horn layers of the beak. Hematoxylin and eosin.

tally oriented and are nearly identical between the two plates. However, the two plates differ in the cells of the intermediate and transitional layers. The intermediate layer of the dorsal plate is similar to that of the beak epidermis with polyhedral cells that generally retain distinct nuclei. In contrast, the intermediate cells of the ventral plate are compacted horizontally into spindle shapes, have weakly eosinophilic contents, and contain flattened, central nuclei. The thick transitional layer of the dorsal plate is composed of cells with diffuse nuclear material and eosinophilic intercellular laminae. In the ventral plate, distinct nucleoli are retained into the transitional layer, which has strongly eosinophilic intercellular laminae with clear or absent cytoplasm. The stratum transitivum of the ventral plate more closely resembles that of soft-cornified skin with a lacy, woven appearance. The stratum corneum of the ventral plate demonstrates intermediate staining characteristics; it is much more eosinophilic than the pale cornified epidermal layer of the dorsal plate of the beak rhamphotheca, but not as dark as collagen or other connective tissue. In contrast, the transitional cells of the dorsal plate merge abruptly into the pale, denucleated stratum corneum. At the junction of the dorsal and ventral plates, there is a sharp ridge that resembles the tomial edge of the beak, with a column of transitional cells that protrudes into the stratum corneum. In the claw, this transition is more abrupt than in the beak and is a site of increased fraying and shedding of the cornified laminae.

DISCUSSION

Techniques

Results from histological sampling of hard-cornified epidermal structures are occasionally presented in the published literature, but few sources report detailed methods. These tissues pose unique

challenges for histological processing due to the variable density and hardness of their components (Lucas and Stettenheim, 1972; Pass, 1989; Homberger et al., 2009). Here, we review the efficacy of our techniques and provide suggestions that may be of use for future studies.

For beak and claw tissue, at least in the case of small passerines, decalcification with formic acid solution combined with post-treatment of embedded tissues with Nair™ appears to be adequate for basic histological sectioning and assessment. Prior to our study, we conducted pilot tests to determine the length of time required for decalcification with formic acid and to ensure good staining quality. Although acids can be damaging to specimens, particularly with regard to altering nuclear staining properties, formic acid is relatively slow-acting, and we did not encounter any apparent problems with a 72-h treatment of beaks and claws. Chelating agents, which require a much longer decalcification period, may also be used and generally result in minimal alteration to staining properties for bone and other tissues (Carson, 1997). However, these compounds can damage proteoglycans and may affect staining of cartilage (Callis and Sterchi, 1998), which could be an issue for assessment of beak tissues. Nair™ contains thioglycolate salts, which helps to break disulfide bonds in keratin-rich tissues. This product has been recognized informally among veterinarians and histopathologists as an effective softening agent for nails, hooves, and other hard-cornified tissues (Immunohistochemistry World, <http://www.ihcworld.com>; Histonet, www.histonet-search.com/histonet; J. L. Oaks pers. comm.), but to our knowledge ours is the first published account of this approach for sectioning avian beaks and claws.

We used paraffin as the embedding medium, which allowed for sectioning with a regular microtome and generally worked well for beak and claw

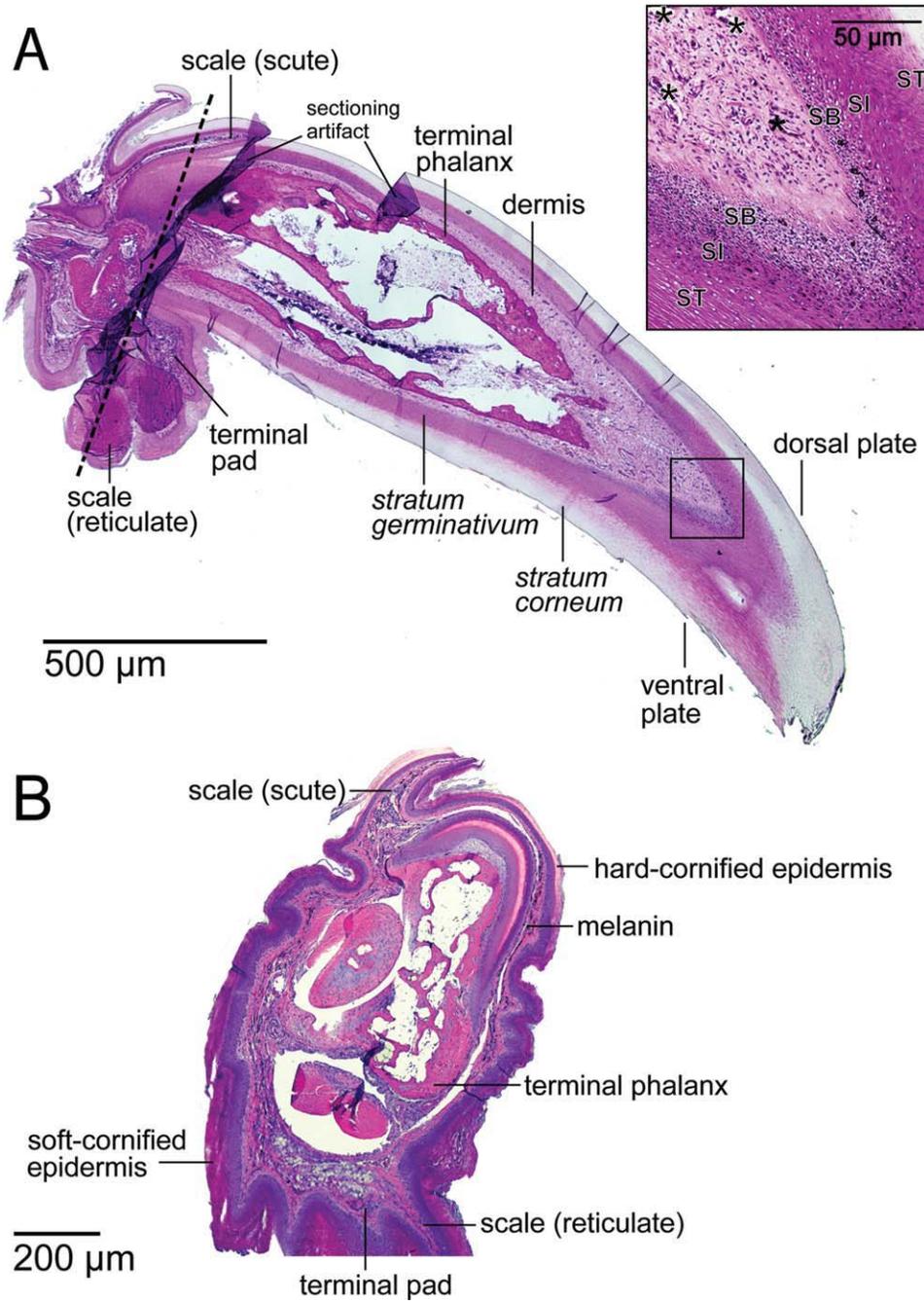


Fig. 10. (A) Mid-sagittal section of claw of Black-capped Chickadee showing bone, dermis, epidermis, scales, and terminal pad. The stratum basale (SB), stratum intermedium (SI), and stratum transitivum (ST) of the dorsal and ventral plates are visible in inset. Capillaries (asterisks) are abundant in the dermis. (B) Transverse section of claw near the base. Dashed line in (A) shows approximate plane of section in (B). Hematoxylin and eosin.

tissue in this and other reported studies, but alternative methods should also be considered (Lucas and Stettenheim, 1972; Gentle et al., 1995; Monroe et al., 2003). When resectioning blocks that had been cut previously and stored for up to 1 year, we encountered increased fracturing of hard-cornified tissues, resulting in poorer sections. These problems were likely due in part to increased dehydra-

tion of tissues, which may have occurred despite the sealing of cut surfaces with paraffin prior to storage. Improved climate control of storage areas and possible thermal (Shlopov et al., 1984) or hydration (Shapiro, 1978) treatment of blocks prior to sectioning could help remedy this problem. Alternatively, a higher melting-point paraffin would provide an embedding medium that more

closely matches the hardness of beak and claw tissues and may increase consistency of results (Lucas and Stettenheim, 1972). Other studies have reported using resin instead of paraffin (Homberger and Brush, 1986; Alibardi, 2002; Homberger et al., 2009), which apparently sections well but presents additional processing challenges and can interfere with some staining properties (Bancroft and Gamble, 2008).

Basic staining of avian beaks with hematoxylin and eosin demonstrated most major structures in the chickadee beaks and claws we examined and has been reported by others to reveal histopathological changes associated with certain disease conditions (Kingsbury et al., 1953; Lucas and Stettenheim, 1972; Homberger and Brush, 1986; Gentle et al., 1995; O'Toole and Raisbeck, 1997; Monroe et al., 2003). However, we also found that special stains greatly improved our ability to distinguish specific features of interest. The stratum corneum of the beak epidermis stained most distinctly with Masson's trichrome which, along with PAS, also revealed the basement membrane. Masson's trichrome and PTAH stains were best for demonstrating Herbst corpuscles, connective tissue, and bone. As expected, respiratory structures, including cartilage of the nasal passages, and salivary glands were easiest to view with PAS (Carson, 1997). Polarized light allowed us to detect columns in the beak epidermis that were otherwise only partially visible using normal light microscopy. We found that mid-sagittal sections typically produced higher quality slides than the more easily fractured transverse sections. However, a combination of mid-sagittal and transverse sectioning provided the greatest opportunity to sample all structures of interest. Detection of some microanatomical structures is only possible with special stains or techniques, and we therefore recommend using a multifaceted approach when dealing with cornified tissues (see also Homberger et al., 2009).

Form and Function

This study of two major hard-cornified structures of the Black-capped Chickadee provides a baseline description of the normal, healthy microanatomy of the passerine beak and claw. As expected, many of the basic structures we observed are typical of these tissues, but there are also unique characteristics that distinguish the beak and claw of the Black-capped Chickadee from those of other species.

Evaluation of our histology images shows that the chickadee rhamphotheca is thicker relative to the overall size of the beak than that of the chicken (*Gallus gallus*), the species for which the most comparable published images are available (Lucas and Stettenheim, 1972; Lunam, 2005; Kuenzel, 2007). Although precise comparisons are

difficult without exact measurements or identical planes of section, the hard-cornified layer of the chickadee epidermis appears to be more similar in proportion to that of several other free-ranging species: the Great-spotted Woodpecker (*Dendrocopus major*), Bohemian Waxwing (*Bombycilla garrula*; Lüdicke, 1933), European Oystercatcher (*Haematopus ostralegus*; Heppleston, 1970), and some psittacines (Menzel and Lüdicke, 1974). A thick rhamphotheca capable of sustaining high and variable rates of wear may be necessary for species that use their beaks to forage on hard substrates or otherwise incur significant mechanical wear. Like many other cavity-nesting species, chickadees exert considerable stress on their beaks by hammering on wood and tree bark and excavating nest sites. As dietary generalists, their feeding behaviors include probing beneath tree bark for insects, opening rigid seed husks, and pounding on hard, frozen food items in winter (Foote et al., 2010). The hard-cornified layer of the chickadee claw also appears to be proportionally thicker, relative to its overall size, than that of the chicken, the only species for which detailed images are available (Lucas and Stettenheim, 1972). Localized thickening of the hard-cornified epidermis of the chickadee beak (at the tips and along the tomium) and claw (at the tip and along the juncture of dorsal and ventral plates) may help to compensate for intense wear in these regions.

The architecture of the cornified layer of the chickadee rhamphotheca reflects normal growth and wear patterns of the beak epidermis. Overlapping corneocytes on the surface of the rhamphotheca are elongated longitudinally, which is likely a product of the dominant proximal to distal growth of the beak epidermis (Menzel and Lüdicke, 1974; Seki et al., 2010). We observed a more variable orientation and a reduced adhesion of the individual corneocytes at sites of increased wear and tear along the tip and tomial edges of the beak. A clear junction between the inner and outer horn layers is visible with SEM and polarized light microscopy and extends through the full thickness of the stratum corneum, suggesting the presence of a structurally significant transition with disruption of the cornified laminae. Similarly, Lüdicke (1933) reported discontinuity of laminae between the inner and outer surfaces of the beak in a variety of species. However, this interpretation contrasts with a description of the beak of the chicken by Lucas and Stettenheim (1972), who noted the existence of lateral and medial columns of transitional cells but asserted that the layers of the stratum corneum continue uninterrupted over the tomial edges. It is possible that interspecific variation may explain this discrepancy, although differences in processing or viewing techniques could also affect interpretation.

We observed the highest density of melanin granules in the cornified layer of the superficial and tomial surfaces of the beak, a pattern which likely serves both mechanical and behavioral functions. Due to the cross-linking properties of its large polymers, melanin adds strength to integumentary tissues and may reduce the effects of mechanical wear (Bonser and Witter, 1993; Stettenheim, 2000; McGraw, 2006). Thus, the preferential incorporation of melanin granules into the chickadee rhamphotheca could further harden the hard-cornified epidermis in regions of increased wear (Bonser and Witter, 1993). In addition, melanin pigmentation, which is responsible for the beak's dark color, plays a role in social signaling and its expression in the integument of Black-capped Chickadees has been associated with dominance, reproductive status, and physiological condition (Mennill et al., 2003; Doucet et al., 2005; Woodcock et al., 2005). Accordingly, melanin granules are especially abundant in the superficial and, therefore, visible regions of the chickadee rhamphotheca.

As expected, we observed Herbst corpuscles throughout the dermis of the chickadee beak and claw, but only singly or in small clusters. These mechanoreceptors were less densely packed than those observed in other species reported to have a bill tip organ, including various shorebirds, ducks, geese, and parrots (Krogis, 1931; Zweers and Wouterlood, 1973; Berkhoudt, 1976; Gentle and Breward, 1986; Piersma et al., 1998; Lunam, 2005; Cunningham et al., 2007, 2010). In accordance with reports from other seed-eating passerine species, chickadees do not display features of a bill tip organ (Ziswiler and Trnka, 1972; Krulis, 1978). This specialized structure has been associated with the degree to which the beak is used for finding or manipulating food (Gottschaldt, 1985). Chickadees are highly visual foragers and use their beaks primarily for mechanical functions such as excavating nest cavities or opening seeds that generally do not require extensive processing (Foote et al., 2010). The infrequent occurrence of Grandry corpuscles, movement-sensitive mechanoreceptors (Gottschaldt, 1985), in the chickadee beak may be similarly related to the rarity of tactile foraging in this species. Our study was not intended to serve as a comprehensive assessment of mechanoreceptors in the chickadee beak and claw and more detailed future investigation of these structures could be aided by specific techniques, such as the use of a silver stain (Piersma et al., 1998; Cunningham et al., 2007, 2010).

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