EPIZOOTIC OF BEAK DEFORMITIES IN ALASKA: INVESTIGATION OF AN EMERGING AVIAN DISEASE

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EPIZOOTIC OF BEAK DEFORMITIES IN ALASKA: INVESTIGATION OF AN EMERGING AVIAN DISEASE

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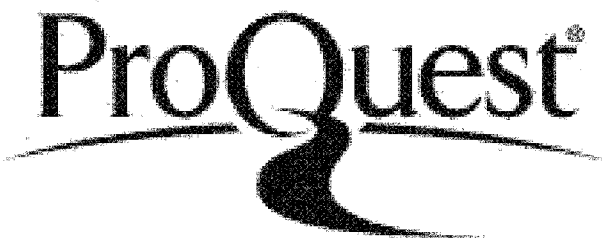

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ABSTRACT

The sudden appearance of morphological abnormalities in a wild population is often associated with underlying ecological disturbances, including those related to introduction of new pathogens or pollutants. An epizootic of beak deformities recently documented among Black-capped Chickadees (*Poecile atricapillus*) and other resident bird species in Alaska has raised concern about underlying causes. This dissertation describes results from several recent studies of what we have termed "avian keratin disorder." The primary objectives of the research were to characterize the physiology and pathology of beak deformities and to address specific ecological questions related to this emerging avian disease. In a study of beak growth rates in captive chickadees, I determined that accelerated epidermal growth is the primary physical mechanism by which beak deformities develop and are maintained. Affected birds also exhibited high rates of mortality and skin lesions, suggesting that this disorder significantly compromises individual health. I used radiography, histopathology, and electron microscopy to describe the pathology of avian keratin disorder. As part of this effort, I established baseline information about normal passerine beak and claw structure and developed methods for processing hard-cornified tissues. The suite of lesions that I observed in affected chickadees does not correspond with any known avian diseases, suggesting the presentation of a novel disorder in wild birds. In addition, the detailed characterization of gross and microscopic changes has allowed me to eliminate a number of likely etiologies, including nutritional problems, microbial pathogens, and select toxicants. As a complement to diagnostic pathology, I conducted field studies to

investigate possible causes and patterns of occurrence of beak deformities. I used stable isotope analysis to investigate the association between diet and beak deformities. I found that winter dietary patterns differed between chickadees with normal beaks and those with beak deformities, but that such differences are more likely a result than a cause of avian keratin disorder. My field research on Northwestern Crows in Alaska confirmed high prevalence of a nearly identical condition to that observed in chickadees. These findings indicate that avian keratin disorder affects multiple, ecologically-distinct species across a large geographic area. Together, the studies presented in this dissertation provide new insights and identify priority research areas for a rapidly emerging disease in wild birds.

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INTRODUCTION

Beginning in the late 1990s, reports from biologists and the public generated attention about an apparent outbreak of beak abnormalities in Black-capped Chickadees (*Poecile atricapillus*) in south-central Alaska (Handel et al. 2006). Numbers of observations increased exponentially during the ensuing decade and included an expanding geographic area (Handel et al. 2006). Detailed study of chickadees in Alaska subsequently revealed the highest rates of gross abnormalities ever reported in a wild bird population and signaled the emergence of a significant avian epizootic (Handel et al. 2006; Handel et al. 2010). Morphologically similar deformities, characterized by gross overgrowth of the beak, appeared in other resident avian species over the same period, raising concern about underlying environmental factors in this region.

The sudden appearance of morphological abnormalities or gross lesions in a wild population is often associated with underlying ecological disturbances, including those related to introduction of new pathogens or pollutants (e.g., Ohlendorf et al. 1986; Ludwig et al. 1996; Daszak et al. 2001; Dobson and Foufopoulos 2001; Johnson et al. 2007). Beak deformities are rare among adult birds, and a normal, background level of deformities is less than one half of one percent (Pomeroy 1962; Craves 1994). Suspected and known causes of such deformities include infectious disease, parasites, genetic abnormalities, environmental contaminants, and nutritional deficiencies, although precise mechanisms are often difficult to determine (Harrison 1986; Tully et al. 2000; Handel et al. 2010).

Initial efforts to understand the cause of Alaskan beak deformities included screening for toxicants in affected chickadees and their food sources. None of the compounds known to cause beak abnormalities in other species appeared obviously to be implicated in the Alaskan deformities (Handel et al. 2006). Specimens and samples were submitted for necropsy, diagnostic testing, and clinical chemistry. Affected birds did not test positive for known avian diseases and necropsy results were inconclusive (Handel et al. 2006; Handel et al. 2010). Detailed field studies also addressed possible ecological correlates and examined the fitness consequences of these debilitating deformities (Handel et al. 2006). Despite these efforts, preliminary analyses did not reveal the underlying cause of what we have since termed "avian keratin disorder" and many unanswered questions remained.

The significant knowledge gaps associated with this emerging epizootic in wild birds piqued my scientific interest and soon became the focus of my Ph.D. research. This dissertation presents the results of several studies that have helped to enhance our understanding of avian keratin disorder. The first section (Chapters 1–3) evaluates the physiology and pathology of this disorder in Black-capped Chickadees. The second section (Chapters 4–5) addresses ecological questions using field studies of Black-capped Chickadees and Northwestern Crows (*Corvus caurinus*).

Chapter 1 presents results from a captive study in which I measured rates of beak growth in affected and unaffected chickadees. This research was prompted by the need to understand how beak deformities develop and are maintained in affected birds. The rhamphotheca, the outer cornified layer of the beak, is continually replaced through

normal processes of growth and wear (Stettenheim 2000). Thus, the beak overgrowth characteristic of birds affected by avian keratin disorder could be due to accelerated growth, reduced wear, or a combination of these two processes (Handel et al. 2010). In this chapter, I evaluate the hypothesis that gross deformities result from accelerated epidermal growth and describe changes in beak morphology over time.

In order to assess the pathology of avian keratin disorder, I first needed a reference of "healthy" tissues against which to compare cellular abnormalities in affected birds. However, little information is available in the published literature on the microanatomy of avian hard-cornified tissues, which pose significant technical challenges for histologic processing. Chapter 2 addresses these information gaps by providing a description of the passerine beak and claw, using the Black-capped Chickadee as a model. Here I also report the development and application of novel methods to prepare hard-cornified tissues for microscopic examination.

Using this baseline information on normal beaks and claws, I investigated the pathology of avian keratin disorder in Black-capped Chickadees. Chapter 3 describes results from radiography, histology, and electron microscopy. These techniques are important diagnostic tools in veterinary medicine and allow for detection of microscopic and ultrastructural changes in tissues and cells. By screening for characteristic lesions associated with specific pathogens or toxicants, it is also possible to identify or rule out likely etiologies. In this study, I focus primarily on keratinized tissues, including the beak and claw, and describe the underlying changes associated with avian keratin

disorder. These results provide inferences about the mechanisms by which beak deformities develop in chickadees.

As a complement to diagnostic pathology, ecological studies offer important insights about possible causes of this disorder. Dietary sources, including anthropogenic foods, provide a plausible route of exposure for environmental contaminants and pathogens. Therefore, I was interested in investigating a potential association between diet and beak deformities in chickadees. In Chapter 4, I use stable isotope analysis to determine whether dietary composition of individuals differs by deformity status. Based on these patterns, I discuss whether such differences are more likely to be causal factors or functional consequences of deformities.

In order to determine whether reports of beak deformities in other species signaled the emergence of a multi-species epizootic or were simply isolated instances of beak abnormalities, I measured the rate of beak deformities in Alaskan crow populations. Chapter 5 examines prevalence and geographic patterns of beak deformities in Northwestern Crows. In addition, I compare patterns of occurrence between chickadees and crows, which occupy distinct habitats and feeding niches. This cross-species approach helps to define the ecological scope of this problem.

The complexity of avian keratin disorder has prompted a multi-disciplinary investigation using a variety of techniques and methods. Together, these five chapters provide a diverse yet complementary suite of insights about a rapidly emerging disease in wild birds.

CHAPTER 1. EVIDENCE OF ACCELERATED BEAK GROWTH ASSOCIATED WITH AVIAN KERATIN DISORDER IN BLACK-CAPPED CHICKADEES (*POECILE ATRICAPILLUS*)[1]

1.1 ABSTRACT

We recently documented an epizootic of beak deformities in more than 2,000 Black-capped Chickadees (*Poecile atricapillus*) and other wild bird species in North America. This emerging avian disease, which has been termed "avian keratin disorder," results in gross overgrowth of the rhamphotheca, the outer keratinized layer of the beak. To test the hypothesis that the beak deformities characteristic of this disorder are associated with accelerated keratin production, we measured rates of beak growth and wear in affected Black-capped Chickadees (n=16) and a control sample of unaffected chickadees (n=14) collected from south-central (61°09' to 61°38'N, 149°11' to 149°48'W) and interior Alaska (64°51' to 64°53'N, 147°49' to 147°59'W). Rates of absolute growth were 50–100% higher in affected birds than controls and exceeded records from other passerine species. These results suggest that abnormally rapid epidermal growth is the primary physical mechanism by which beak deformities develop and are maintained in affected chickadees. Although beak overgrowth typically worsened over time, differential patterns of wear influenced the severity and morphology of deformities. In some cases, the effects of accelerated keratin growth were partially mitigated by frequent breakage of rhamphothecal tips. However, mortalities occurred in 9 of 16 birds with

[1] Van Hemert, C., C. M. Handel, and T. M. O'Hara. *In press.* Evidence of accelerated beak growth associated with avian keratin disorder in Black-capped Chickadees (*Poecile atricapillus*). *Journal of Wildlife Diseases.*

beak deformities during the study period, suggesting that avian keratin disorder results in severe health consequences for affected birds. Additional study of factors that control beak keratin production is needed to understand the pathogenesis of this debilitating disease in wild birds.

1.2 INTRODUCTION

Abnormalities of the rhamphotheca, the outer cornified layer of the beak, have been documented in a wide range of species (Pomeroy, 1962; Craves, 1994) and may be associated with certain disease conditions, nutritional disorders, and exposure to environmental contaminants, although specific mechanisms are generally not well understood (Harrison, 1986; O'Hara and Rice, 1996; Tully et al., 2000; Keymer and Samour, 2008). An emerging epizootic of beak deformities among wild birds in Alaska, other regions of North America (Handel et al., 2010; Van Hemert and Handel, 2010), and, more recently, Europe (Harrison, 2011), presented the need for detailed investigation of beak growth in a passerine species. Adult Black-capped Chickadees (*Poecile atricapillus*) in Alaska exhibit high prevalence (6.5%) of deformities and have been the subjects of ongoing research on the ecology and pathology of what we have termed "avian keratin disorder" (Handel et al., 2010). Affected birds have elongated, sometimes crossed beaks that compromise their ability to feed and preen (Handel et al., 2010; Van Hemert and Handel, 2010). Preliminary research suggested that these abnormalities primarily affect the rhamphotheca, with no apparent defects in the underlying bone (Handel et al., 2010). In field studies of affected chickadee populations, we observed

very rapid onset of beak deformities in some individuals, with rates of net growth far exceeding those reported for other passerine species (Handel et al., 2010). Increased thickness and brittleness and irregularities in surface appearance were also suggestive of abnormal epidermal growth (Handel et al., 2010). Therefore, we suspected that rapid production of keratin was contributing to beak overgrowth, but this hypothesis had not yet been tested.

In a normal beak, patterns of growth and wear are typically well balanced, allowing the rhamphotheca to maintain a consistent length and shape that closely matches that of the underlying skeletal structure (Campàs et al., 2010). The germinative layer of the beak epidermis grows continually, producing keratinized cells that become incorporated into the rhamphotheca as they mature. This growth process is mediated by behaviors such as feeding and pecking that result in mechanical wear and subsequent sloughing of the external cornified layers (Stettenheim, 2000). It is not known whether growth rates are typically constant over time, with variability in wear and abrasion leading to seasonal changes in beak morphology (Clancey, 1948; Davis, 1954; Morton and Morton, 1987), or if growth may fluctuate in response to hormonal, physical, or other potential stimuli. Thus, the beak overgrowth characteristic of birds affected by avian keratin disorder could be due to accelerated growth, reduced wear, or a combination of these two processes (Handel et al., 2010).

The primary objective of this study was to test the hypothesis that beak deformities in Black-capped Chickadees are associated with accelerated keratin production. To do so, we compared rates of absolute beak growth between affected birds

and a control sample of unaffected birds. We also assessed the role that beak wear plays in the development of these deformities by calculating rates of net growth and wear in the two groups.

1.3 Materials and Methods

1.3.1 Field methods

In autumn 2008, we used funnel traps and mist nets (Handel et al., 2010) to capture adult Black-capped Chickadees from south-central (61°09' to 61°38'N, 149°11' to 149°48'W) and interior Alaska (64°51' to 64°53'N, 147°49' to 147°59'W). Through a targeted trapping effort, we captured 16 individuals (8 males, 8 females) with various forms and severity of beak deformities using the criteria established by Handel et al. (2010). We selected 14 birds (7 males, 7 females) with normal beaks as controls. We aged birds by plumage characteristics (Pyle, 1997) and used molecular techniques to determine sex from blood samples drawn from the brachial vein (Handel et al., 2006; Handel et al., 2010).

1.3.2 Captive experiment

After transport to the University of Alaska Fairbanks (UAF) Biological Research and Diagnostic Facility, birds were individually housed in 76 cm x 46 cm x 46 cm stainless steel cages and maintained at 10° C under full-spectrum lights set to the photoperiod of Anchorage, Alaska (61°10'N, 149°59'W), adjusted weekly to match natural seasonal changes in daylight period. Diet consisted of chipped sunflower seeds,

suet, mealworms, and ground hard-boiled eggs provided *ad libitum* in low-rimmed petri dishes. Avian Calcium for Birds (Zoo Med Laboratories, Inc.) and Avi-Con avian multivitamin (Vet-A-Mix, LLOYD, Inc.) were provided according to manufacturer guidelines. All work was completed under guidance of the UAF and the U. S. Geological Survey Alaska Science Center Institutional Animal Care and Use committees (UAF assurance no. 08-57).

We examined the beak and took a series of seven measurements (with digital calipers to 0.1 mm) every 14 days between December 9, 2008, and April 13, 2009. On each occasion, we lightly etched beaks with a scalpel blade (see Wydoski, 1964; Hulscher, 1985; Matthysen, 1989) at two locations (proximal, distal) on the upper beak (approximately 3 and 5 mm distal of the nares) and one location on the lower beak (approximately 3 mm distal of the juncture of the rami at the base of the gonys). We measured the chord distance from the anterior end of the right nare or the base of the gonys to the respective markings for absolute growth and to the tip of the upper (nares-to-tip) or lower beak (gonys) beak for net growth. We also measured the chord of the upper beak beyond where it meets the lower beak (overbite) and the chord of the lower beak beyond the upper (underbite). All measurements were taken by a single individual (CV) throughout the study. During or shortly after the pre-trial quarantine period, three birds with severe beak deformities died, reducing the initial sample size of affected birds to 13 (6 males, 7 females).

1.3.3 Statistical analyses

We calculated the daily rate of absolute beak growth during each time period by dividing the difference between previous and current measurements by number of days elapsed. We conduct a repeated-measures mixed-model analysis of growth rates at each of the three locations, with individual identity as a random effect (PROC MIXED; SAS 9.1). We included a categorical fixed effect for disorder status (affected versus control) and linear and quadratic fixed effects for time of season (measured at 14-day intervals), along with attendant interaction terms. We reduced the initial saturated model by dropping non-significant terms ($P>0.05$) in a backward-stepwise procedure and then tested the reduced model for differences in marginal means between affected and control groups. We used a paired t-test to determine if absolute growth rates differed between the proximal and distal regions of the upper beak for individuals in either the control or affected groups.

For each bird, we calculated the mean daily rate of net beak growth by dividing the cumulative change in beak length (nares-to-tip and gonys) by total number of days between the bird's first and last measurements. To estimate the mean daily rates of beak wear, we subtracted total net growth from total absolute growth (sum of absolute change at the proximal upper beak or lower beak location), and divided by total number of days. We then used a t-test with the Sattherthwaite adjustment for unequal variances to compare rates of net growth and wear between control and affected birds. Beak tips occasionally broke off in individuals with severe deformities, which introduced an intermittent, catastrophic form of wear distinct from the usual gradual, abrasion-driven

wear process. To address this issue, we used a general linear model with post-hoc Tukey-Kramer comparisons and tested for differences in net growth and wear between controls, affected individuals with breakage, and affected individuals without breakage.

We were also interested in whether the amount of abrasion-related beak wear during a given time period might stimulate absolute beak growth during the subsequent period. To test this, we created a model with absolute growth rates of the upper (proximal measurement) and lower beaks as response variables and included terms from the best-fitting model from the initial repeated-measures analysis. We then added a lag term for beak wear (upper or lower) during the previous time period, excluding those in which breakage occurred, to determine if previous beak wear explained any significant amount of variation in absolute beak growth.

Finally, we used mean beak length of each bird to assess the relative effect of the severity of deformity on beak wear. We regressed mean rate of wear (excluding periods with breakage) against mean beak length over the course of the study, with one data point per individual.

We conducted analyses in SAS Version 9.1; all probabilities are two-tailed and means are presented ±SE.

1.4 RESULTS

1.4.1 Beak morphology and observations

Affected birds displayed a variety of beak deformity morphologies that affected the upper beak, the lower beak, or both (Fig. 1.1). At the time of capture, affected birds

(n=16) had a mean nares-to-tip length of 11.6±1.2 mm (6.8–25.2 mm) and gonys length of 9.2±1.2 mm (6.7–24.1 mm), with overbite of 4.2±1.4 mm (0–16.7 mm) and underbite of 1.4±1.0 mm (0–15.0 mm). Three affected birds had crossed upper and lower beaks at the time of capture and an additional nine affected birds later developed varying degrees of lateral deviation and crossing. In all cases, the severity of the deformity remained the same or worsened over time, with net increase in the upper and lower beak of up to 3.5 and 2.7 mm per month, respectively. Breakage of the beak tips was relatively common in extremely elongated beaks, which reduced the deformity length and sometimes resulted in a more normal appearance of the beak. However, the beak typically returned to a similarly deformed state within 2–6 weeks.

Control birds (n=14) had a mean nares-to-tip length of 7.3±0.1 mm (6.8–7.6 mm) and gonys length of 6.7±0.1 mm (5.8–7.0 mm) at the time of capture. We observed only minor overbite (0.1±0.02 mm) and no underbite. Slight changes in beak morphology (<1.0 mm) occurred among several individuals in the control group, but these changes were typically transient and beaks regained their original shape and size within several weeks.

Nine of 16 (56.3±12.4%) affected birds died during the study. Mortalities occurred between 5 and 105 days after introduction to captivity and happened most frequently among individuals with severe beak deformities. In contrast, none of the control birds died (χ^2=11.2, df=1, P=0.001). Affected birds also displayed other signs of poor health, including skin lesions, which will be described in detail elsewhere.

1.4.2 Beak growth

The upper beak grew nearly twice as rapidly in affected birds (n=13) as in controls (n=14), with marginal mean absolute growth rates of 0.115±0.006 versus 0.070±0.002 mm/day for the proximal region ($t_{18.7}$=-7.26, P<0.001) and 0.120±0.007 mm/day versus 0.069±0.002 mm/day for the distal region ($t_{15.8}$=-7.26, P<0.001; Fig. 1.2). The rate of absolute growth of the lower beak was about 50% greater in affected birds (0.094±0.004 mm/day) than in controls (0.065±0.001 mm/day; $t_{16.3}$=-7.36, P< 0.001; Fig. 1.2). Time of season had a slight but significant effect on absolute growth rates, with different patterns for control and affected birds (Table 1.1, Fig. 1.2).

The variable lag(wear) was not a significant predictor of absolute growth for either the upper beak ($F_{1,135}$=1.77, P=0.186) or lower beak ($F_{1,151}$=0.02, P=0.886), suggesting no relationship between the amount of beak wear and subsequent growth rate. Mean rates of absolute growth were the same for the proximal and distal regions of the upper beak for control birds (t_{13}=0.28, P=0.783) and differed only marginally for affected birds (t_{12}=-1.94, P=0.076).

For controls, mean net growth rates were equivalent to zero for both the upper beak (0.002±0.001 mm/day) and lower beak (0.002±0.002 mm/day). For affected birds overall, net growth rates for the upper beak (0.024±0.012 mm/day) and lower beak (0.018±0.009 mm/day) were higher but statistically indistinguishable from controls (upper beak: t_{13}=0.28, P=0.783; lower beak: t_{12}=-1.94, P=0.076). However, net growth rates differed significantly between affected individuals with breakage and those without (Fig. 1.3). The resulting groups (control, affected with breakage, affected without

breakage) differed significantly for both the upper ($F_{2,26}$=13.48, P<0.001) and lower ($F_{2,26}$=41.28, P<0.001) beak. Net growth in affected birds with breakage was approximately zero for both the upper and lower beak (nares to tip: -0.002±0.009 mm/day, n=7; gonys: 0.001±0.003 mm/day, n= 9) and did not differ from that in controls (nares to tip: P=0.091, gonys: P=0.970). In contrast, net growth in affected birds without breakage was significantly higher than in controls for both the upper beak (0.054±0.017 mm/day; P<0.001, n=6) and the lower beak (0.058±0.012 mm/day; P<0.001, n=4; Fig. 1.3).

Rates of beak wear showed the same pattern, with no significant differences between controls and all affected birds combined (upper beak: $t_{13.1}$=1.79, P=0.097; lower beak: $t_{12.9}$=1.5, P=0.157). However, when affected birds were split according to the presence or absence of breakage, rates of wear differed significantly between the three groups for both the upper beak ($F_{2,26}$=19.50, P<0.001) and lower beak ($F_{2,26}$=33.47, P<0.001). Rates of wear in affected individuals with breakage (upper beak: 0.125±0.014 mm/day; lower beak: 0.095±0.004 mm/day) were higher than those in controls (upper beak: 0.068±0.003 mm/day; lower beak: 0.063±0.002 mm/day) or affected birds without breakage (upper beak: 0.054±0.011 mm/day; lower beak: 0.035±0.001 mm/day; all P<0.001). Mean rates of wear were lower for affected birds without breakage than for controls, but this difference was only significant for the lower beak (P=0.003). The rate of abrasion-driven wear decreased as mean beak length increased for both the upper beak (R^2_{adj}=0.873, $F_{2,13}$=5.94, P=0.033) and the lower beak (R^2_{adj}=0.885, $F_{2,13}$=12.35, P=0.005).

1.5 Discussion

Our findings support the hypothesis that abnormally rapid epidermal growth, and not lack of wear, is the primary means by which beak deformities develop and are maintained in Black-capped Chickadees. Rates of absolute beak growth were 50–100% faster in affected birds relative to controls, and exceeded records for all other passerines (Lüdicke, 1933; Wydoski, 1964; Menzel and Lüdicke, 1974; Matthysen, 1989; Table 1.2) and some non-passerines (Lüdicke, 1933; Menzel and Lüdicke, 1974; Hulscher, 1985; Table 1.2). Affected chickadees also showed much greater variability in rates and patterns of beak growth than controls. Our results from unaffected birds indicated that the beak epidermis normally grows at a consistent rate and at a similar speed in the upper and lower beaks, which concurs with measurements from other species (Lüdicke, 1933; Menzel and Lüdicke, 1974; Hulscher, 1985). In contrast, growth rates of affected birds varied and typically corresponded with the severity and the morphology of the deformity. We also detected a statistically marginal, but potentially biologically significant, difference between rates of growth at the proximal and distal regions of the upper beak for affected individuals, a trend that may reflect irregular rates or pulses of accelerated growth. The seasonal pattern of growth rates in affected birds differed from controls, which is likely related to variability in growth rates within individuals as well as changes in the sample population over the study period due to mortalities.

We found that the effects of accelerated keratin growth may be partially mitigated by beak wear in some individuals. Fracturing of beak tips, a catastrophic and intermittent form of wear, occurred at least once in most affected birds and often resulted in

significant changes in beak length. Reduction in natural beak wear via abrasion also affected the severity of deformities, but the magnitude of this effect was relatively small given the frequency of breakage. Although captive conditions do not exactly match chickadees' natural environment and rates of breakage may differ between the two settings, we have observed a similar phenomenon among free-ranging birds with beak deformities, which might help to explain the surprising persistence of some severely affected individuals in the wild (Handel et al., 2010).

In the absence of extreme, compensatory wear caused by breakage, beak length increased quickly in affected birds. Given the maximum rate of net growth observed in this study, an average beak could more than double in length over a period of 10 to 12 weeks. It is also important to note that because deformed beaks often become curved as they increase in length, our estimate of net growth using a straight chord measurement was conservative and thus reflected only a minimum net increase. The potential for high rates of net growth corresponds with the rapid development of deformities that we have documented in some wild birds (Handel et al., 2010). Rates of abrasion-driven wear also declined with increased beak length, which presumably resulted from altered use and lack of normal apposition of the beak tips (Lumeij, 1994).

Results from control birds in this study provide insights about normal beak growth, a process that has not been well documented in the literature. Our failure to detect a relationship between amount of beak wear and absolute growth rates suggests that vascular stimulation via mechanical use does not affect beak keratin production, which is consistent with previous research (Hulscher, 1985). We also documented a

slight but significant seasonal decrease in growth rates in control birds for both the upper and lower beak between early winter and late spring. This pattern has been observed inconsistently in other studies of absolute growth (Wydoski, 1964; Hulscher, 1985; Matthysen, 1989).

Although the population-level effects of these widespread deformities are still under investigation, the high mortality rate in captive chickadees suggests that avian keratin disorder severely compromises the health of affected birds. Black-capped Chickadees typically tolerate captive conditions well (Foote et al., 2010) and mortalities are rarely reported in other laboratory studies of this species. Mortalities occurred over the duration of our study, indicating that affected birds were in poor health not only upon entering captivity from the wild, but even after the introduction of an easily accessible, *ad libitum* diet and removal of natural environmental stressors.

The evaluation of possible etiologies has proven to be challenging, but evidence of accelerated beak growth provides an important insight about this disorder. Among birds, certain viral, bacterial, and fungal infections can result in abnormal keratin growth and share some features consistent with avian keratin disorder (e.g., Tully et al., 2000; Schmidt et al., 2003; Stewart et al., 2006). However, most conditions that affect the beak result in different clinical signs from what we have observed in chickadees (Handel et al., 2010), and only limited research has been conducted on avian hard-cornified tissues (review in Van Hemert et al., 2011). Detailed histopathology and other diagnostic tests are currently underway to identify possible mechanisms and likely causative agents of this rapidly emerging avian disease.

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1.7 LITERATURE CITED

CAMPÀS, O., R. MALLARINO, A. HERREL, A. ABZHANOV, AND M. P. BRENNER. 2010. Scaling and shear transformations capture beak shape variation in Darwin's finches. Proceedings of the National Academy of Sciences 107: 3356-3360.

CLANCEY, P. A. 1948. Seasonal bill variation in Tree-Sparrow. British Birds 41: 115-116.

CRAVES, J. A. 1994. Passerines with deformed bills. North American Bird Bander 19: 14-18.

DAVIS, J. 1954. Seasonal changes in bill length of certain passerine birds. Condor 56: 142-149.

FOOTE J. R., D. J. MENILL, L. M. RATCLIFFE, AND S. M. SMITH. 2010. Black-capped Chickadee (*Poecile atricapillus*). *In* Birds of North America, A. POOLE (ed.). Cornell Lab of Ornithology, Ithaca, New York.

HANDEL, C. M., L. M. PAJOT, S. M. MATSUOKA, C. VAN HEMERT, J. TERENZI, S. L. TALBOT, D. M. MULCAHY, C. U. METEYER, AND K. A. TRUST. 2010. Epizootic of beak deformities among wild birds in Alaska: an emerging disease in North America? Auk 127: 882-898.

HANDEL, C. M., L. M. PAJOT, S. L. TALBOT, AND G. K. SAGE. 2006. Use of buccal swabs for sampling DNA from nestling and adult birds. Wildlife Society Bulletin 34: 1094-1100.

HARRISON, G. J. 1986. Disorders of the integument. *In* Clinical Avian Medicine and Surgery, G. J. HARRISON and L. R. HARRISON (eds.). W. B. Saunders Co., Philadelphia, Pennsylvania. pp. 397-407

HARRISON, T. 2011. Beak deformities of garden birds. British Birds 104:138-141.

HULSCHER, J. B. 1985. Growth and abrasion of the Oystercatcher bill in relation to dietary switches. Netherlands Journal of Zoology 35: 124-154.

KEYMER, I. F., AND J. SAMOUR. 2008. Disorders of the digestive system. *In* Avian Medicine, J. SAMOUR (ed.). Elsevier, New York. pp. 281-302

LÜDICKE, M. 1933. Wachstum und abnutzung des vogelschnabels. Zoologische Jahrbücher, Abteilung für Anatomie und Ontogenie der Tiere 57: 435-564.

LUMEIJ, J. T. 1994. Gastro-enterology. *In* Avian Medicine: Principles and Application, B. W. RITCHIE, G. J. HARRISON, and L. R. HARRISON (eds.). Wingers Publishing, Inc., Lake Worth, Florida. pp.

MATTHYSEN, E. 1989. Seasonal variation in bill morphology of Nuthatches *Sitta europaea*: dietary adaptations or consequences? Ardea 77: 117-125.

MENZEL, R., AND M. LÜDICKE. 1974. Funktionell-anatomische und autoradiographische Untersuchungen am Schnabelhorn von Papageien (Psittaci). Zoologische Jahrbücher, Abteilung für Anatomie und Ontogenie der Tiere 93: 175-218.

MORTON, M. L., AND G. A. MORTON. 1987. Seasonal changes in bill length in summering mountain White-Crowned Sparrows. Condor 89: 197-200.

O'HARA, T. M., AND C. D. RICE. 1996. Polychlorinated biphenyls. *In* Noninfectious Diseases of Wildlife, A. FAIRBROTHER, L. N. LOCKE, and G. L. HOFF (eds.). Iowa State University Press, Ames, Iowa. pp. 71-86

POMEROY, D. E. 1962. Birds with abnormal bills. British Birds 56: 49-72.

PYLE, P. 1997. Identification Guide to North American Birds: A Compendium of Information on Identifying, Ageing, and Sexing 'Near-Passerines' and Passerines in the Hand. Slate Creek Press, Bolinas, CA.

SCHMIDT, R. E., D. R. REAVILL, AND D. N. PHALEN. 2003. Pathology of Pet and Aviary Birds. 1st edition. Iowa State Press, Ames, Iowa.

STETTENHEIM, P. R. 2000. The integumentary morphology of modern birds - an overview. American Zoologist 40: 461-477.

STEWART, M. E., R. PERRY, AND S. R. RAIDAL. 2006. Identification of a novel circovirus in Australian Ravens (*Corvus coronoides*) with feather disease. Avian Pathology 35: 86-92.

TULLY, T. N., JR., M. P. C. LAWTON, AND G. M. DORRESTEIN (eds.) 2000. Handbook of Avian Medicine. 2nd edition. Butterworth-Heinemann, Elsevier Science Ltd., New York.

VAN HEMERT, C., AND C. M. HANDEL. 2010. Beak deformities in Northwestern Crows: evidence of a multispecies epizootic. Auk 127: 746-751.

VAN HEMERT, C., C. M. HANDEL, J. E. BLAKE, R. M. SWOR, AND T. M. O'HARA. 2011. Microanatomy of passerine hard-cornified tissues: beak and claw structure of the Black-capped Chickadee (*Poecile atricapillus*). Journal of Morphology 273: 226-240.

WYDOSKI, R. S. 1964. Seasonal changes in the color of starling bills. Auk 81: 542-550.

Table 1.1 Results of repeated-measures mixed-model analysis on the effect of avian-keratin-disorder status (affected vs. control) and time of season (linear or quadratic) on keratin growth at three locations on the beak for Black-capped Chickadees in captivity between December 2008 and April 2009.

Variable	Degrees of freedom	*F*	*P*
Upper beak			
Proximal			
Avian-keratin-disorder status	2, 132	91.7	<0.001
Time of season	1, 116	3.7	0.058
(Time of season)^2	1, 113	5.8	0.017
Status x Time	1, 116	6.0	0.016
Status x (Time)^2	1, 113	4.5	0.036
Distal			
Avian-keratin-disorder status	2, 129	116.5	<0.001
Time of season	1, 102	1.45	0.231
(Time of season)^2	1, 99.7	3.33	0.071
Status x Time	1, 102	4.7	0.032
Status x (Time)^2	1, 99.7	4.07	0.046
Lower beak			
Avian-keratin-disorder status	2, 129	100.8	<0.001
Time of season	1, 118	0.15	.699
Status x Time	1, 118	4.17	0.043

Table 1.2 Rates of absolute growth (mm/day) measured in the upper beak of passerine and non-passerine species.

Species	*n*	Mean growth rate (mm/day)	Captive/Wild	Source
Passerines				
Poecile atricapillus (Black-capped Chickadee; normal beak)	14	0.07	Captive	This study
P. atricapillus (Black-capped Chickadee; beak deformity)	13	0.12	Captive	This study
Sitta europaea (Eurasian Nuthatch)	45	0.09	Wild	Matthysen 1989
Sturnus vulgaris (European Starling)	14	0.07	Captive	Wydoski 1964
Serinus canaria (Common Canary)	1	0.08	Captive	Lüdicke 1933
Coccothraustes coccothraustes (Hawfinch)	1	0.03	Captive	Menzel and Lüdicke 1974
Non-passerines				
Haematopus ostralegus (Eurasian Oystercatcher)	12	0.27[1]	Captive	Hulscher 1985
H. ostralegus (Eurasian Oystercatcher)	10	0.44	Wild	Hulscher 1985
Columba livia domestica (Domestic Pigeon)	1	0.05	Captive	Lüdicke 1933
Eos bornea (Red Lory)	1	0.06	Captive	Menzel and Lüdicke 1974
Melopsittacus undulatus (Budgerigar)	1	0.15	Captive	Menzel and Lüdicke 1974
Agapornis roesicollis (Rosy-faced Lovebird)	1	0.13	Captive	Menzel and Lüdicke 1974
Ara ararauna (Blue-and-yellow Macaw)	1	0.11	Captive	Menzel and Lüdicke 1974
Dendrocopos major (Great Spotted Woodpecker)	1	0.38	Captive	Lüdicke 1933

[1]Mean value calculated from raw values presented for two captive groups in Hulscher (1985).

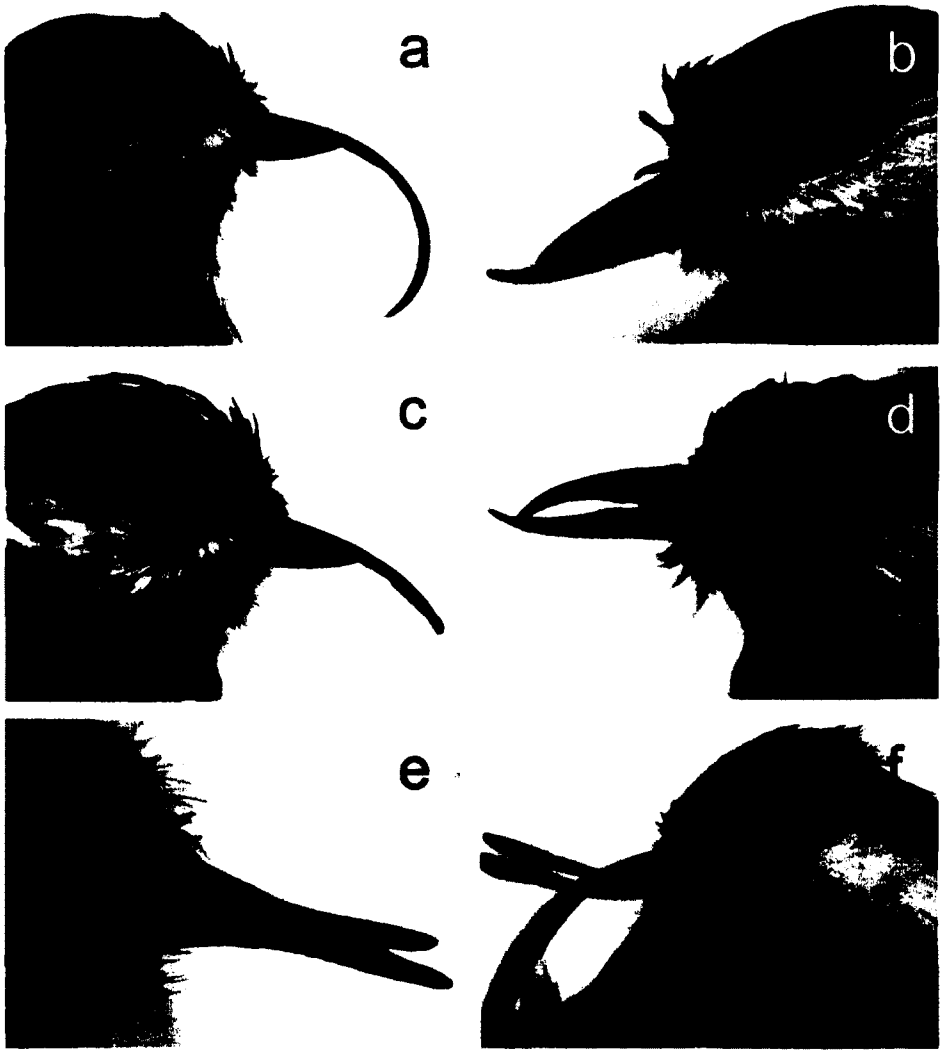

Figure 1.1 Black-capped Chickadees affected by avian keratin disorder displayed various beak-deformity-morphologies: overbite (a, c), underbite (b), elongation of both upper and lower beak (d), and crossing and lateral deviation (e, f). Figure (e) shows ventral view.

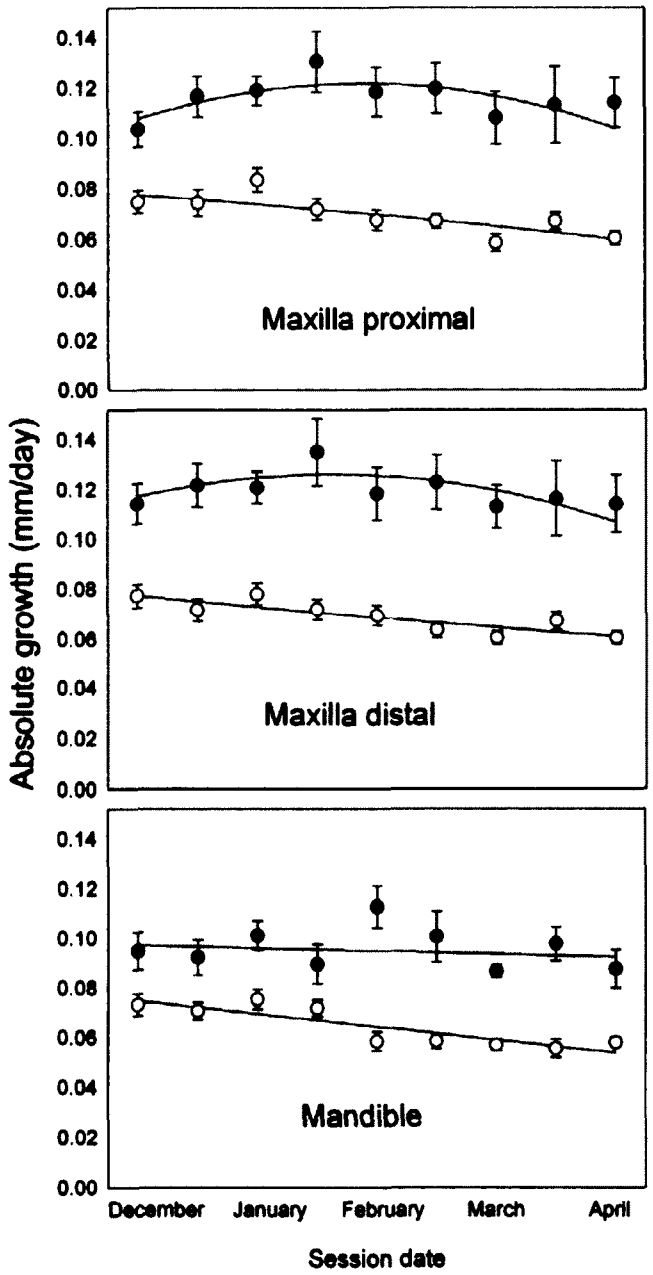

Figure 1.2 Mean (±SE) rates of absolute beak growth (mm/day) measured at three locations on the rhamphotheca between late winter and early spring in captive Black-capped Chickadees affected (black circles) and unaffected (white circles) by avian keratin disorder. Lines show best-fit regressions for each group.

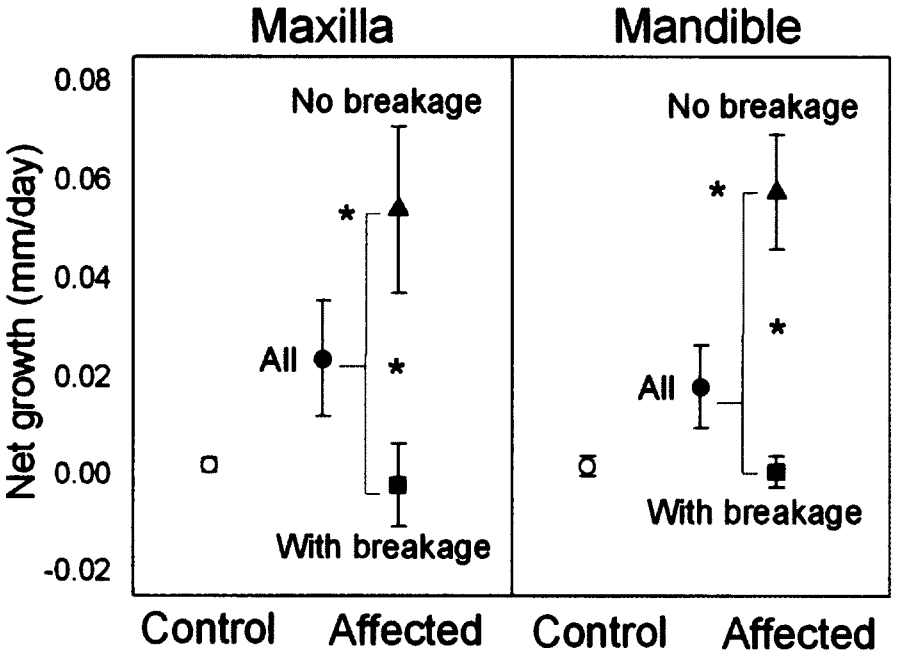

Figure 1.3 Mean (±SE) rate of net growth (mm/day) in length of the upper beak (left panel) and lower beak (right panel) in captive Black-capped Chickadees affected (black symbols) and unaffected (white circles) by avian keratin disorder. Affected birds with no breakage of beak tips (black triangles) had significantly higher rates of net growth than affected birds with intermittent breakage (black squares) and controls. Rate of net growth did not differ significantly between all affected birds combined (black circles) and controls. Net growth was calculated using straight chord from nares to the tip of the upper beak and along gonys of the lower beak

CHAPTER 2. MICROANATOMY OF PASSERINE HARD-CORNIFIED TISSUES: BEAK AND CLAW STRUCTURE OF THE BLACK-CAPPED CHICKADEE (*POECILE ATRICAPILLUS*) [1]

2.1 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 the present 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

[1] Van Hemert, C., C. M. Handel, J. E. Blake, R. M. Swor, and T. M. O'Hara. *In press*. Microanatomy of passerine hard-cornified tissues: Beak and claw structure of the Black-capped Chickadee (*Poecile atricapillus*). *Journal of Morphology*.

will provide a necessary reference for ongoing investigations of avian keratin disorder in Black-capped Chickadees and other wild passerine species.

2.2 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; Van Hemert and Handel, 2010). Our investigation of this disease, termed "avian keratin disorder," has highlighted the need for additional background information on passerine hard-cornified tissues. In order to comprehend the pathology of avian keratin disorder, we must first understand normal microanatomy and structure, a requirement that prompted the current 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 epidermis 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).

Inter-specific differences in beak and claw structure 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; Cunningham et al., 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; Cunningham et al., 2010). Several recent publications have also described notably unusual or exotic beaks and accessories (Homberger, 2001; Seki et al., 2005; Seki et al., 2006; Chen et al., 2008; Tattersall et al., 2009; Seki et al., 2010). 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 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.

2.3 Materials and Methods

2.3.1 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 (~4° 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 U. S. Geological Survey Alaska Science Center Institutional Animal Use and Care committees (Assurance #07-49, 08-57) and complied with all pertinent legal requirements.

2.3.2 Laboratory methods

After necropsy, we fixed whole specimens in 10% neutral buffered formalin for 72 hours 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 20x magnification (40kV, 2.8W) 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 Scanning Electron Microscope. 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 100–1000x 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 hours. 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 mm, 3 mm, 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 *Nair*™ (Church & Dwight Co., Inc.; Princeton, New Jersey) for approximately 5 min, followed by cooling for 5–10 min

on an ice bath prior to sectioning. We cut sections at 5 μm with an American Optical 820 Spencer microtome (American Optical; Buffalo, New York), floated them in a heated (18–30° C) water bath, and mounted them onto charged glass slides (VWR Superfrost Plus Micro Slide; VWR International, LLC; Radnor, Pennsylvania). We repeated the *Nair*™ 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.

2.4 RESULTS

2.4.1 Beak microanatomy

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. 2.1, 2.2, 2.3). 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. 2.2, 2.3). At the tip of the beak, these bones are replaced by wide dermal layers and a thickened epidermis (Figs. 2.1, 2.2, 2.4).

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. 2.5). Eosinophilic connective tissue, including elastin and collagen, surrounds the cartilage, providing additional support to the nasal passages (Fig. 2.5). 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. 2.5D), which is replaced by respiratory epithelium (Fig. 2.5B) in the middle chamber and infraorbital sinus and olfactory epithelium in the caudal chamber. Pseudostratified, ciliated columnar respiratory epithelium with abundant mucous glands is evident in the middle nasal chamber and sinuses (Fig. 2.5B). Olfactory epithelium of the caudal nasal chamber is composed of a single layer of cuboidal cells (Fig. 2.5C). Salivary glands are visible on the buccal sides of both the upper and lower beaks (Fig. 2.5C).

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. 2.2, 2.3, 2.6). 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. 2.3). A similar mirrored arrangement of vessels and nerves is present in the lower beak, with a reversed dorso-ventral orientation relative to the mandibular bone

(Fig. 2.3). 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 (Figs. 2.6B, 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. 2.6). In transverse sections ($n = 5$) taken 1 mm distal to the external nares, the number of Herbst corpuscles in the dermis varied from 5–10 total with clusters of 1–4 near the tomial edges in the upper beak, and 4–6 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. 2.6C).

A thin, PAS-positive basement membrane separates the epidermis from the dermis (Fig. 2.5A, 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. 2.2). 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. 2.2, 2.7). At the tips of the upper and lower beaks, basal cells incline distally and nuclei are flattened into crescent shapes (Fig. 2.6B). 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. 2.2, 2.7). 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 inter-cellular laminae (Figs. 2.2, 2.7). 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.

Cells of the *stratum corneum* are squamous, fully denucleated, and form sheets of hard horn (Figs. 2.2, 2.7). 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. 2.2). 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. 2.8A). 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. 2.8B). Sheets of mature corneocytes appear to conform generally to the shape of the beak (Fig. 2.8C), but exhibit possible fissures at the abrupt corners of the tomial edges (Fig. 2.8D).

Light microscopy also demonstrated cellular differences at the tomial edges of the beak. In transverse sections, we observed lateral and medial columns (Fig. 2.6D), 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. 2.9A). 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. 2.9B). Near the tomial edges of the beak, 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. 2.2, 2.7). 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.

2.4.2 Claw microanatomy

The terminal phalanx underlies the claw and, like the bones of the beak, comprises trabecular bone with large marrow spaces (Fig. 2.10). The fat tissue underlying the terminal pad and reticulate scales and scutes cushion and protect at the base of the claw (Fig. 2.10). 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. 2.10). 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. 2.10A). The basal cells of the *stratum*

germinativum are cuboidal or columnar and distally-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 inter-cellular 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.

2.5 Discussion

2.5.1 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-hour 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.histonetsearch.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 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 one year, we encountered increased fracturing of hard-cornified tissues, resulting in poorer sections. These problems were likely due in part to increased dehydration 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 multi-faceted approach when dealing with cornified tissues (see also Homberger et al., 2009).

2.5.2 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 inter-specific 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; Cunningham et al., 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; Cunningham et al., 2010).

Findings from our study of the Black-capped Chickadee beak and claw offer insights on passerine hard-cornified structures and provide important baseline data for an upcoming disease investigation of avian keratin disorder. By describing detailed methods, we present basic guidelines for histological processing of these tissues. Additional research on hard-cornified tissues in other passerine species will be needed to confirm our results and provide focused inter-specific comparisons of microanatomical form and function of avian beaks and claws.

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2.7 Literature Cited

Abzhanov A. 2010. Darwin's Galapagos finches in modern biology. Philos Trans R Soc Lond B Biol Sci 365:1001-1007.

Alibardi L. 2002. Keratinization and lipogenesis in epidermal derivatives of the Zebrafinch, *Taeniopygia guttata castanotis* (*Aves, Passeriformes, Ploecidae*) during embryonic development. J Morphol 251:294-308.

Badyaev AV. 2010. The beak of the other finch: coevolution of genetic covariance structure and developmental modularity during adaptive evolution. Philos Trans R Soc Lond B Biol Sci 365:1111-1126.

Badyaev AV, Young RL, Oh KP, Addison C. 2008. Evolution on a local scale: developmental, functional, and genetic bases of divergence in bill form and associated changes in song structure between adjacent habitats. Evolution 62:1951-1964.

Bancroft JD, Gamble M. 2008. Theory and Practice of Histological Techniques. Philadelphia: Elsevier Health Sciences. 725 p.

Berkhoudt H. 1976. The epidermal structure of the bill tip organ in ducks. Neth J Zool 26:561-566.

Boag PT, Grant PR. 1981. Intense natural selection in a population of Darwin's finches (*Geospizinae*) in the Galapagos. Science 214:82-85.

Bonser RHC, Witter MS. 1993. The indentation hardness of the bill keratin of the European Starling. Condor 95:736-738.

Bowman RI. 1961. Morphological differentiation and adaptation in the Galapagos finches. Univ Calif Publ Zool 58:1-302.

Callis G, Sterchi D. 1998. Decalcification of bone: literature review and practical study of various decalcifying agents, methods, and their effects on bone histology. J Histotech 21:49-58.

Carson FL. 1997. Histotechnology : A Self-instructional Text. Chicago: ASCP Press. 304 p.

Chen PY, Lin AYM, Lin YS, Seki Y, Stokes AG, Peyras J, Olevsky EA, Meyers MA, McKittrick J. 2008. Structure and mechanical properties of selected biological materials. J Mech Behav Biomed Mater 1:208-226.

Clayton DH, Moyer BR, Bush SE, Jones TG, Gardiner DW, Rhodes BB, Goller F. 2005. Adaptive significance of avian beak morphology for ectoparasite control. Proc Biol Sci 272:811-817.

Cooper JE, Harrison GJ. 1994. Dermatology. In: Ritchie BW, Harrison GJ, Harrison LR, editors. Avian Medicine: Principles and Application. Lake Worth, FL: Wingers Publishing. p 607-639.

Cunningham S, Castro I, Alley M. 2007. A new prey-detection mechanism for kiwi (*Apteryx* spp.) suggests convergent evolution between paleognathous and neognathous birds. J Anat 211:493-502.

Cunningham SJ, Alley MR, Castro I, Potter MA, Cunningham M, Pyne MJ. 2010. Bill morphology of ibises suggests a remote-tactile sensory system for prey detection. Auk 127:308-316.

Doucet SM, Mennill DJ, Montgomerie R, Boag PT, Ratcliffe LM. 2005. Achromatic plumage reflectance predicts reproductive success in male Black-capped Chickadees. Behav Ecol 16:218-222.

Falkenberg G, Fleissner G, Schuchardt K, Kuehbacher M, Thalau P, Mouritsen H, Heyers D, Wellenreuther G, Fleissner G. 2010. Avian magnetoreception: elaborate iron mineral containing dendrites in the upper beak seem to be a common feature of birds. PLoS ONE 5:e9231.

Feduccia A. 1993. Evidence from claw geometry indicating arboreal habits of *Archaeopteryx*. Science 259:790-793.

Fleissner G, Stahl B, Thalau P, Falkenberg G, Fleissner G. 2007. A novel concept of Fe-mineral-based magnetoreception: histological and physicochemical data from the upper beak of homing pigeons. Naturwissenschaften 94:631-642.

Fletcher OJ, Abdul-Aziz T. 2008. Avian Histopathology. Jacksonville, Fla.: American Association of Avian Pathologists. 438 p.

Foote JR, Menill DJ, Ratcliffe LM, Smith SM. 2010. Black-capped Chickadee (*Poecile atricapillus*). In: Poole A, editor. Birds N Am. Ithaca, NY: Cornell Lab of Ornithology.

Gentle MJ, Breward J. 1986. The bill tip organ of the Chicken (*Gallus gallus* var. *domesticus*). J Anat 145:79-85.

Gentle MJ, Thorp BH, Hughes BO. 1995. Anatomical consequences of partial beak amputation (beak trimming) in Turkeys. Res Vet Sci 58:158-162.

Glen CL, Bennett MB. 2007. Foraging modes of Mesozoic birds and non-avian theropods. Curr Biol 17:R911-R912.

Gottschaldt KM. 1974. The physiological basis of tactile sensibility in the beak of geese. J Comp Physiol A 95:29-47.

Gottschaldt KM. 1985. Structure and function of avian somatosensory receptors. In: King AS, McLelland J, editors. Form and Function in Birds. London: Academic Press. p 375-461.

Handel CM, Pajot LM, Matsuoka SM, Van Hemert C, Terenzi J, Talbot SL, Mulcahy DM, Meteyer CU, Trust KA. 2010. Epizootic of beak deformities among wild birds in Alaska: an emerging disease in North America? Auk 127:882-898.

Heppleston PB. 1970. Anatomical observations on the bill of the Oystercatcher (*Haematopus ostralegus occidentalis*) in relation to feeding behaviour. J Zool 161:519-524.

Homberger DG. 2001. The case of the cockatoo bill, horse hoof, rhinoceros horn, whale baleen, and turkey beard: the integument as a model system to explore the concepts of homology and non-homology. In: Dutta HM, Datta Munshi JS, editors. Vertebrate Functional Morphology: Horizon of Research in the 21st Century. Enfield, New Hampshire: Science Publishers. p 317-343.

Homberger DG, Brush AH. 1986. Functional-morphological and biochemical correlations of the keratinized structures in the African Grey Parrot, *Psittacus erithacus* (Aves). Zoomorphology 106:103-114.

Homberger DG, Ham K, Ogunbakin T, Bonin JA, Hopkins BA, Osborn ML, Hossain I, Barnett HA, Matthews II KL, Butler LG, Bragulla HH. 2009. The structure of the cornified claw sheath in the domesticated cat (*Felis catus*): implications for the claw-shedding mechanism and the evolution of cornified digital end organs. J Anat 214:620-643.

Kingsbury JW, Allen VG, Rotheram BA. 1953. The histological structure of the beak in the chick. Anat Rec 116:95-115.

Krogis A. 1931. On the topography of Herbst's and Grandry's corpuscles in the adult and embryonic duck bill. Acta Zoologica 12:241-263.

Krulis V. 1978. Struktur und Verteilung von Tastrezeptoren im Schnabel-Zungenbereich von Singvögeln, im besonderen der Fringillidae. Rev Suisse Zool 85:385-447.

Kuenzel WJ. 2007. Neurobiological basis of sensory perception: welfare implications of beak trimming. Poult Sci 86:1273-1282.

Landmann A, Winding N. 1995. Guild organisation and morphology of high altitude granivorous and insectivorous birds. Oikos 73:237-250.

Lucas AM, Stettenheim PR. 1972. Avian Anatomy: Integument. Agriculture Handbook 362. Washington, D. C.: U.S. Department of Agriculture.

Lüdicke M. 1933. Wachstum und abnutzung des vogelschnabels. Zool Jahrb Abt Anat Ontog Tiere 57:435–564.

Luna G, editor. 1968. AFIP Manual of Histologic Staining Techniques. 3rd ed. New York: McGraw-Hill Publications. 258 p.

Lunam CA. 2005. The anatomy and innervation of the Chicken beak: effects of trimming and re-trimming. In: Glatz PC, editor. Poultry Welfare Issues: Beak Trimming. Nottingham, U.K.: Nottingham University Press. p 51-68.

McGraw KJ. 2006. Mechanics of melanin-based coloration. In: Hill GH, McGraw KJ, editors. Bird Coloration: Mechanisms and Measurements. Cambridge, MA: Harvard University Press. p 243-294.

Mennill DJ, Doucet SM, Montgomerie R, Ratcliffe LM. 2003. Achromatic color variation in Black-capped Chickadees, *Poecile atricapilla*: black and white signals of sex and rank. Behav Ecol Sociobiol 53:350-357.

Menzel, Lüdicke M. 1974. Funktionell-anatomische und autoradiographische Untersuchungen am Schnabelhorn von Papageien (Psittaci). Zool Jahrb Abt Anat Ontog Tiere 93:175-218.

Monroe AD, Latimer KS, Pesti GM, Bakalli RI. 2003. Pathology and histology of dietary tryptophan deficiency in broiler chicks. Avian Dis 47:1393-1398.

Moyer BR, Clayton DH. 2004. Avian defenses against ectoparasites. In: van Emden HF, Rothschild M, editors. Insect and Bird Interactions. Andover, U.K.: Intercept. p 241-247.

O'Toole D, Raisbeck MF. 1997. Experimentally induced selenosis of adult Mallard ducks: clinical signs, lesions, and toxicology. Vet Pathol 34:330-340.

Olsen GH. 2003. Oral biology and beak disorders of birds. Vet Clin N Am Exot Anim Pract 6:505-521.

Partridge L. 1976. Some aspects of the morphology of Blue Tits (*Parus caemleus*) and Coal Tits (*Parus ater*) in relation to their behaviour. J Zool 179:121-133.

Pass DA. 1989. The pathology of the avian integument: a review. Avian Pathol 18:1 - 72.

Pass DA, Perry RA. 1984. The pathology of psittacine beak and feather disease. Aust Vet J 61:69-74.

Piersma T, Van Aelst R, Kurk K, Berkhoudt H, Maas LRM. 1998. A new pressure sensory mechanism for prey detection in birds: the use of principles of seabed dynamics? Proc R Soc Biol Sci Ser B 265:1377-1383.

Schmidt RE, Reavill DR, Phalen DN. 2003. Pathology of Pet and Aviary Birds. Ames, Iowa: Iowa State Press. 234 p.

Seki Y, Bodde SG, Meyers MA. 2010. Toucan and hornbill beaks: a comparative study. Acta Biomater 6:331-343.

Seki Y, Kad B, Benson D, Meyers MA. 2006. The toucan beak: structure and mechanical response. Mater Sci Eng C 26:1412-1420.

Seki Y, Schneider MS, Meyers MA. 2005. Structure and mechanical behavior of a toucan beak. Acta Mater 53:5281-5296.

Shapiro SH. 1978. Postembedding adjuvants in routine microtomy of paraffin-processed tissues. Biotech Histochem 53:269-272.

Shlopov VG, Krivenko NA, Shevchenko TI. 1984. Modification of the method of preparation of paraffin blocks. Arkh Patol 86:80-81.

Soons J, Herrel A, Genbrugge A, Aerts P, Podos J, Adriaens D, de Witte Y, Jacobs P, Dirckx J. 2010. Mechanical stress, fracture risk and beak evolution in Darwin's ground finches (Geospiza). Philos Trans R Soc Lond B Biol Sci 365:1093-1098.

Stettenheim PR. 2000. The integumentary morphology of modern birds—an overview. Am Zool 40:461-477.

Tattersall GJ, Andrade DV, Abe AS. 2009. Heat exchange from the toucan bill reveals a controllable vascular thermal radiator. Science 325:468-470.

Van Hemert C, Handel CM. 2010. Beak deformities in Northwestern Crows: evidence of a multispecies epizootic. Auk 127:746-751.

Woodcock EA, Rathburn MK, Ratcliffe LM. 2005. Achromatic plumage reflectance, social dominance and female mate preference in Black-capped Chickadees (*Poecile atricapillus*). Ethology 111:891-900.

Yalden DW. 1985. Forelimb function in *Archaeopteryx*. In: Hecht MK, Ostrom JH, Viohl G, Wellnhofer P, editors. The Beginnings of Birds. Eichstätt: Jura-Museum. p 91-97.

Yasui K, Hayashi Y. 1967. Morphogenesis of the beak of the chick embryo: histological, histochemical and autoradiographic studies. Embryologia (Nagoya) 10:42-74.

Zelená J, Halata Z, Szeder V, Grim M. 1997. Crural Herbst corpuscles in chicken and quail: numbers and structure. Anat Embryol 196:323-333.

Ziswiler V, Trnka V. 1972. Tastkörperchen im Schlundbereich der Vögel. Rev Suisse Zool 79:307-318.

Zweers GA, Wouterlood FG. 1973. Functional anatomy of the feeding apparatus of the Mallard (*Anas platyrhynchos L.*). In: Proceedings of the Third European Anatomical Congress. Manchester, U. K. p 88-89.

Figure 2.1 High-magnification radiograph showing underlying bone structure of the beak in relation to external margins of the hard-cornified epidermis of the rhamphotheca (rhinotheca, gnatotheca) in Black-capped Chickadee.

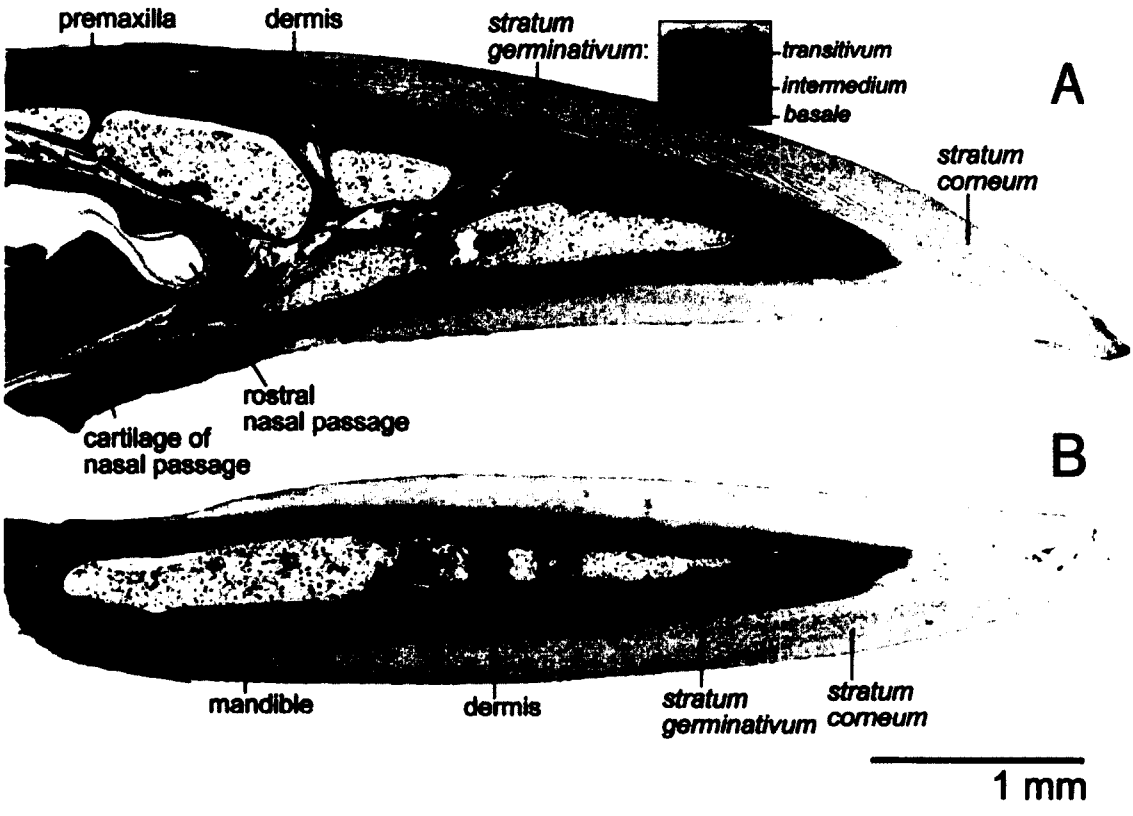

Figure 2.2 Mid-sagittal section of (A) upper and (B) lower beak of Black-capped Chickadee showing underlying bone core (premaxilla, mandible), dermis, epidermis (*stratum germinativum, stratum corneum*), and nasal passages. Hematoxylin and eosin.

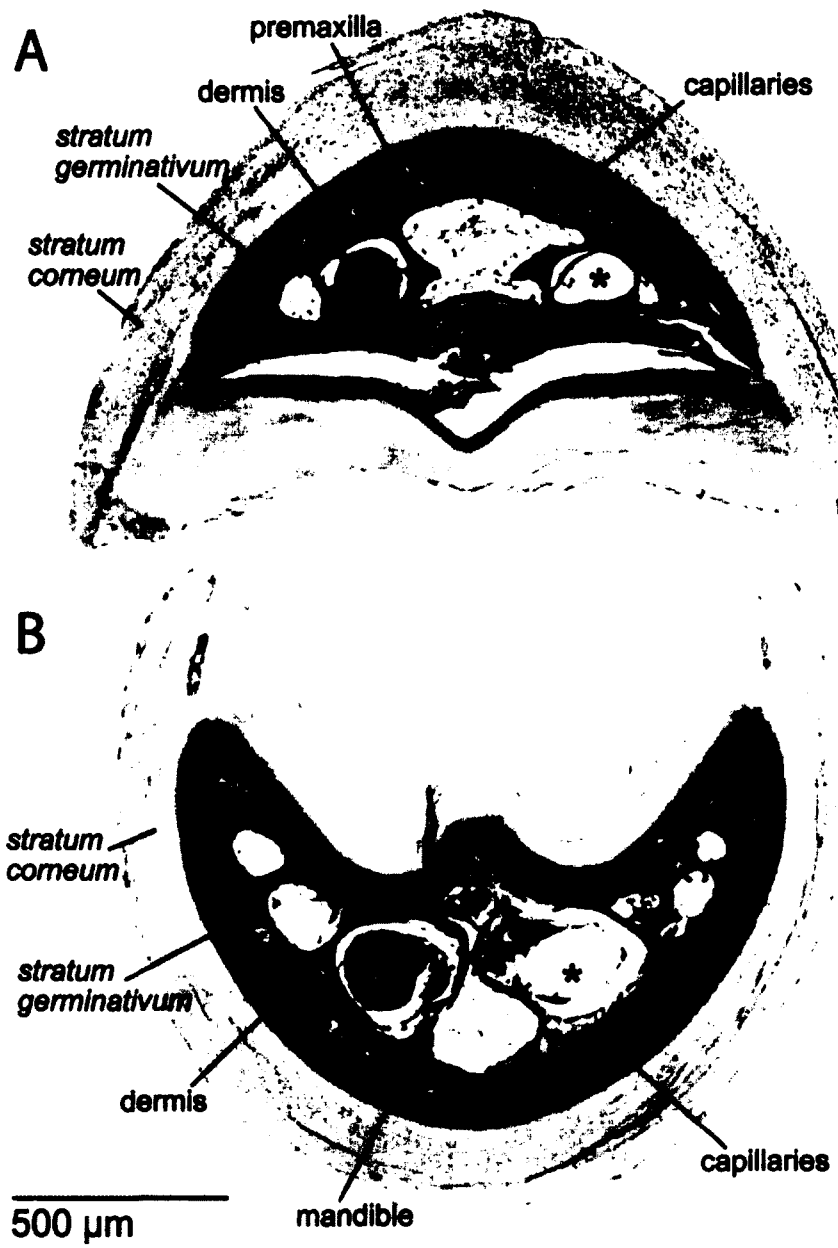

Figure 2.3 Transverse sections 1 mm distal to external nares of upper (A) and lower (B) beak of Black-capped Chickadee. Note trabecular bone core and thick dermal and epidermal layers. Channels within trabecular bone house large blood vessels (asterisks) adjacent to bundled arteries (a) and veins (v). Hematoxylin and eosin.

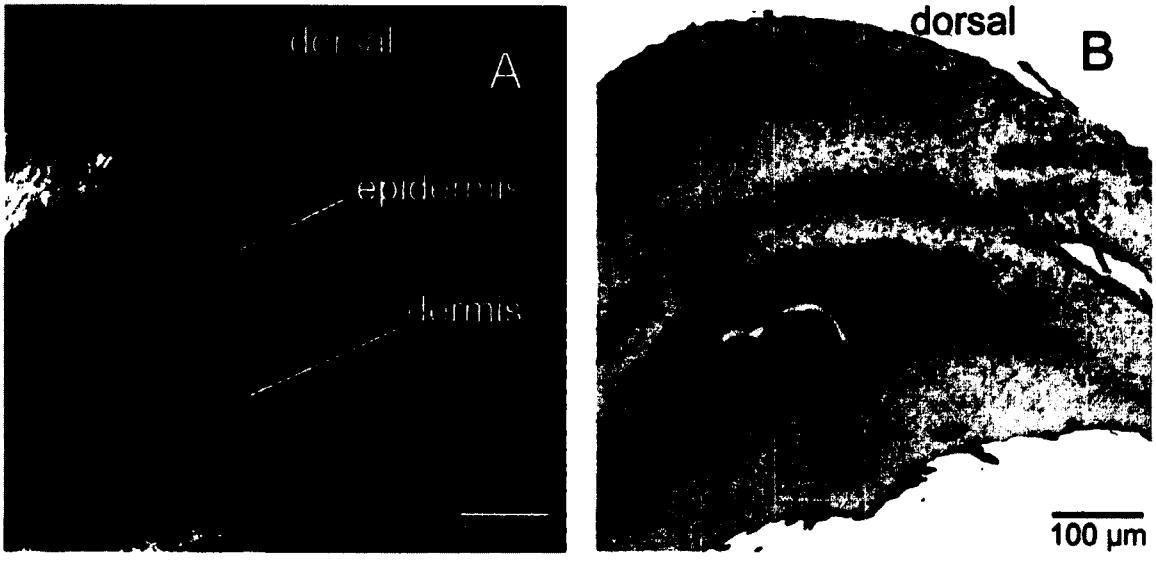

Figure 2.4 Transverse sections of upper beak of Black-capped Chickadee near its tip. Scanning electron (A) and light (B, hematoxylin and eosin) microscopy demonstrate lack of bone core and thickened dermis and epidermis, including cornified (*corneum*) and germinative (*germ.*) layers, at this distal location.

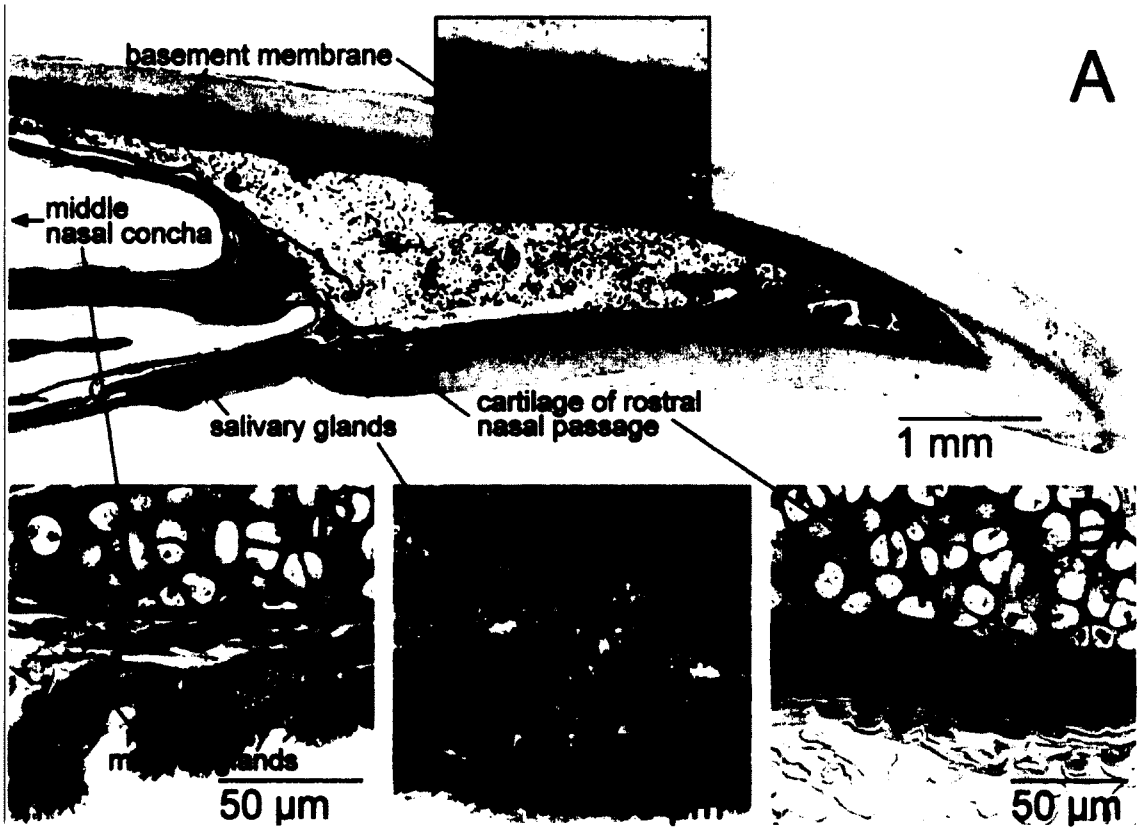

Figure 2.5 Examples of specialized respiratory and olfactory structures structures in upper beak of Black-capped Chickadee: (A) PAS-positive basement membrane, salivary glands, and cartilage of rostral nasal passages; (B) hyaline cartilage, pseudo-stratified, ciliated respiratory epithelium, and mucous 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. Periodic acid Schiff (A), hematoxylin and eosin (B–D).

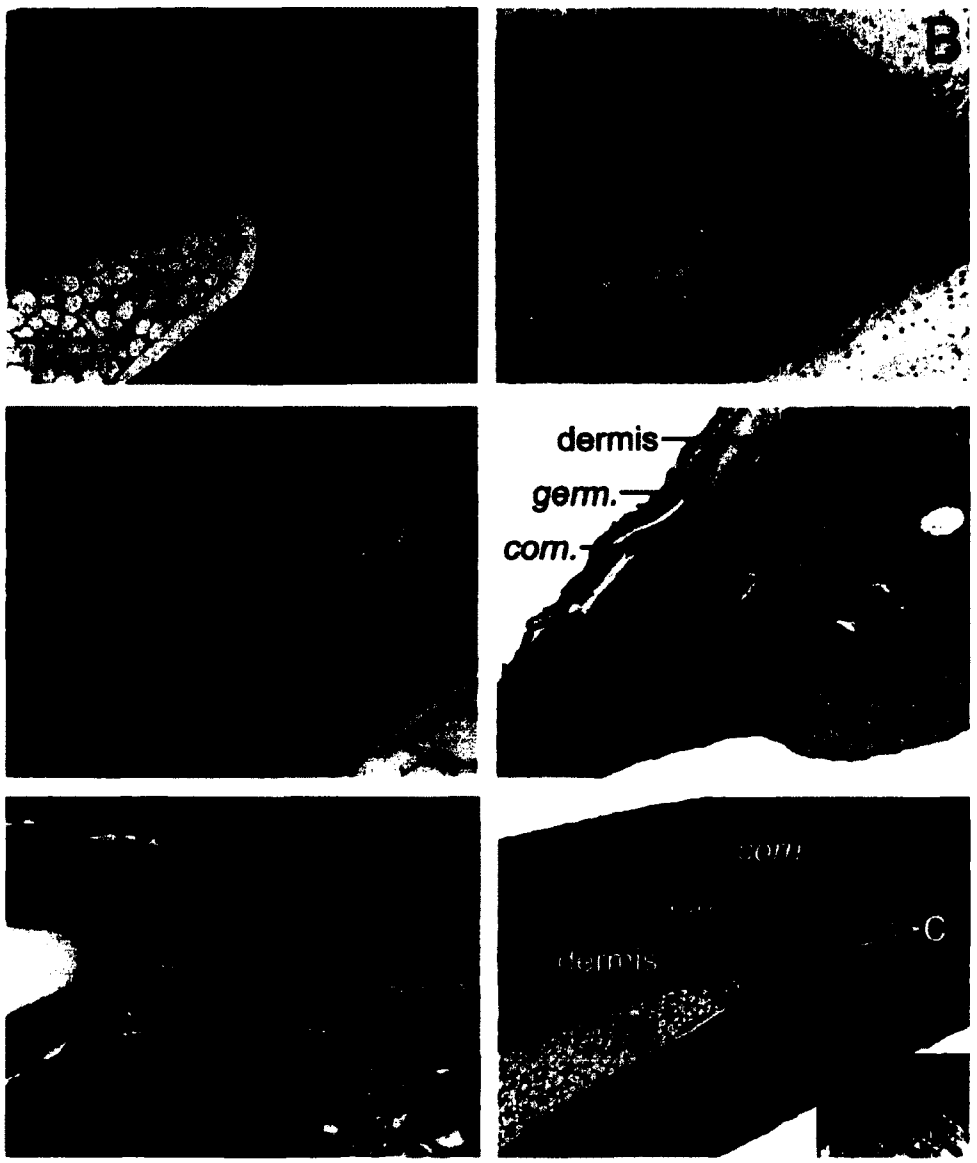

Figure 2.6 Mid-sagittal sections of upper (A, E, F) and lower (B, C) beak and transverse section of lower beak (D) of Black-capped Chickadee showing bone (b), dermis, cornified (*corn.*) and germinative (*germ.*) layers of the epidermis, and lateral and medial columns. Adipocytes are visible within the 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), phosphotungstic acid hematoxylin (E), Masson's trichrome (F).

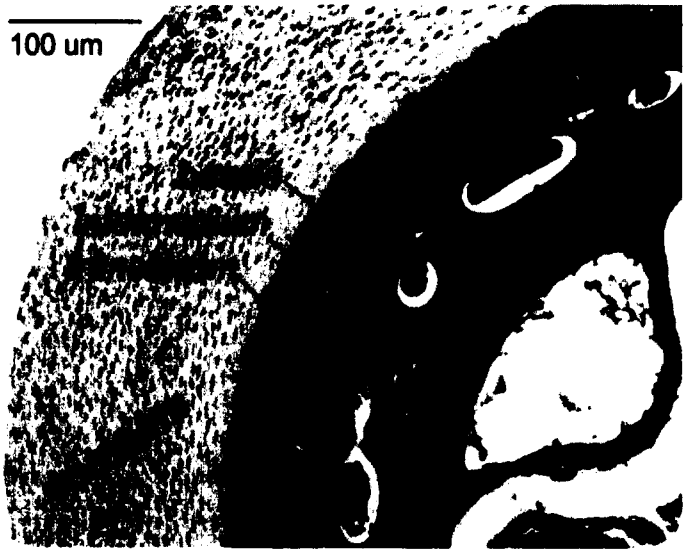

Figure 2.7 Transverse section of upper beak of Black-capped Chickadee showing bone, dermis, and cornified (*corneum*) and germinative (*germinativum*) layers of the epidermis. Note dense melanin granules in cornified layer. Hematoxylin and eosin.

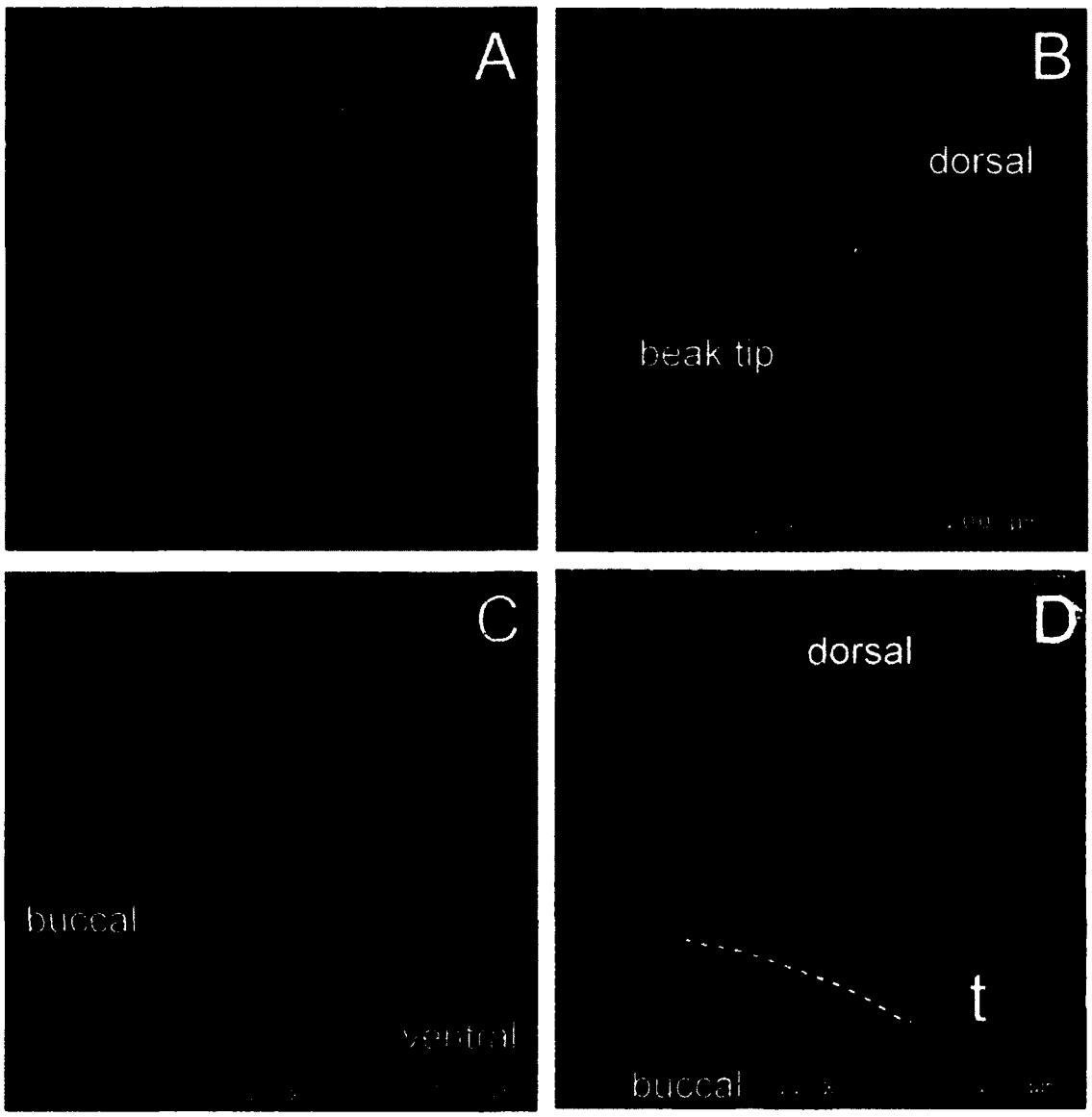

Figure 2.8 Scanning electron micrographs of Black-capped Chickadee beak: (A) flattened, longitudinally-elongated corneocytes on dorsal surface of upper beak; (B) dorsal view of tip of upper beak showing reduced adhesion of corneocytes at the beak tip; (C) transverse section of lower beak showing cornified 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).

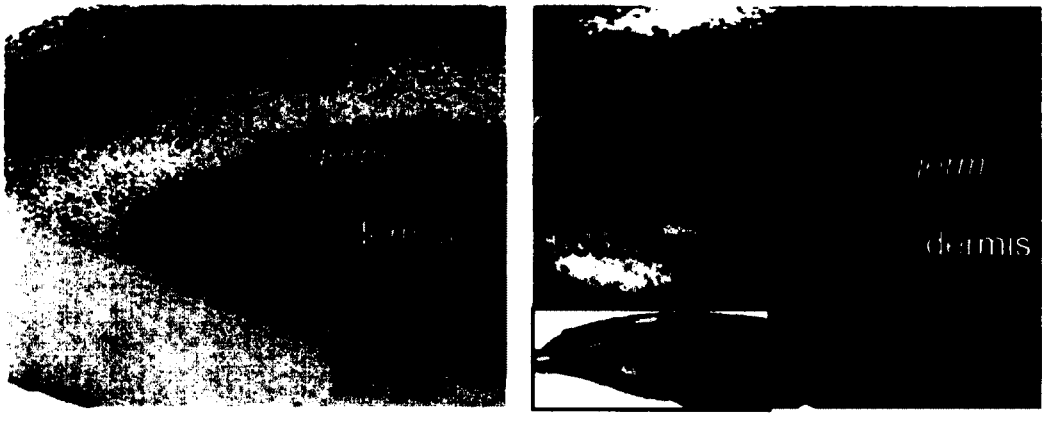

Figure 2.9 Mid-sagittal section of upper beak of Black-capped Chickadee under normal light (A) and polarized light with a lambda filter (B). As shown here, lateral and medial transitional columns (blue in [B]) occur throughout the cornified layer of the beak (inset) but are detectable only with polarized light. These columns may represent a structurally significant junction between the inner and outer horn layers of the beak. Hematoxylin and eosin.

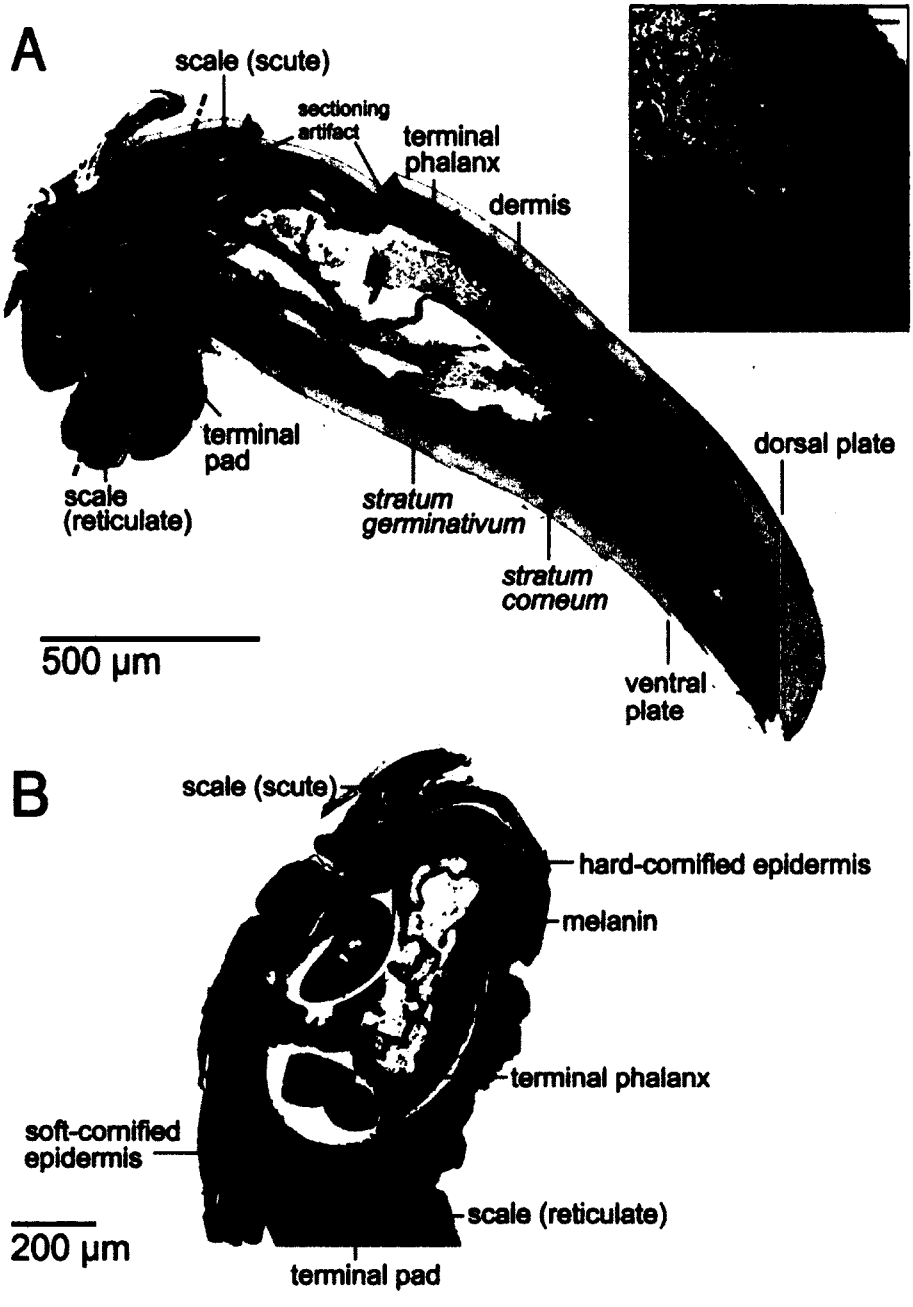

Figure 2.10 Micrographs of claw of Black-capped Chickadee. (A) Mid-sagittal section of claw showing bone, dermis, epidermis, scales, and terminal pad. The *stratum basale* (SB), *stratum intermedium* (SI), and *stratum transitivum* (ST) of the dorsal and ventral plates are visible in inset. Capillaries (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.

CHAPTER 3. PATHOLOGY OF AVIAN KERATIN DISORDER IN BLACK-CAPPED CHICKADEES (*POECILE ATRICAPILLUS*)[1]

3.1 ABSTRACT

An epizootic of beak deformities was recently detected among wild birds in Alaska. Here we describe the gross, histologic, and ultrastructural features of what has been termed "avian keratin disorder" in 30 adult Black-capped Chickadees (*Poecile atricapillus*). Grossly, affected birds presented with mild to severe beak overgrowth, sometimes accompanied by lesions in other keratinized tissues, particularly claws and skin. The most prominent histologic features in the beak included epidermal hyperplasia, hyperkeratosis, and core-like intrusions of necrotic debris. We observed remodeling of maxillary and mandibular bones and various dermal lesions, particularly in birds with moderate to severe beak overgrowth. Claws demonstrated some lesions analogous to those found in beaks, indicating that this disorder may target both of these biochemically and structurally similar tissues. Other keratinized tissues, including skin, feather follicles, and, occasionally, sinus epithelium, exhibited mild to moderate hyperkeratosis, but typically only in the presence of microbial pathogens. Gross and microscopic presentation of other, non-keratinized tissues was unremarkable and we did not find consistent evidence of a bacterial, fungal, or viral etiology. The lesions observed in

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affected birds did not correspond with any known avian diseases, suggesting a potentially novel disorder in wild birds.

3.2 INTRODUCTION

An epizootic of beak deformities, termed "avian keratin disorder", was recently documented in Black-capped Chickadees (*Poecile atricapillus*) and Northwestern Crows (*Corvus caurinus*) in Alaska.[8,31] The estimated prevalence of this disorder in affected populations of these species (6.5% and 16.9%, respectively) exceeds published rates of gross deformities in wild birds.[8] In addition, as many as 28 other, mostly nonmigratory, species in the Pacific Northwest region of North America may be affected.[8] New reports suggest the emergence of a similar disorder in the United Kingdom.[11] The high rate of occurrence in multiple avian species across a growing geographic area raises concern about possible causes, including infectious agents, toxins, or metabolic disorders. The etiology and pathogenesis of this emerging disease are not yet known.

Affected birds display various forms of beak deformities, including elongation, crossing, and lateral deviation, which impair feeding, preening, and other normal behaviors.[8,31] Study of this disease in Alaska has highlighted important fitness consequences.[4,8] Evidence of high mortality rates and reduced reproductive success [8,9,33] suggests the potential for deleterious impacts at the population level. Beak deformities in other species have been attributed to a variety of toxic, nutritional, infectious, and traumatic etiologies, although precise mechanisms are often difficult to determine.[10,30] The underlying pathologic changes associated with beak deformities in Black-capped

Chickadees have not yet been described and it is unknown whether this disease affects multiple keratinized tissues or is primarily restricted to the beak.

Here we document the gross, microscopic, and ultrastructural features of avian keratin disorder in affected Black-capped Chickadees. Our objectives were to characterize the suite of lesions associated with this disorder and to screen for potential pathogens.

3.3 Materials and Methods

3.3.1 Study animals

In spring and fall, 2008, and spring, 2011, we used funnel traps and mist nets to capture adult Black-capped Chickadees from several locations in south-central and interior Alaska.[8] Thirty birds with gross beak deformities that fit the criteria described by Handel et al.[8] were included for examination. We assigned three categories of severity based on the amount of overgrowth of the upper or lower beak (gross nares-to-tip and/or gonyl measurements at necropsy): mild (≤9.0 mm), moderate (9.1–14.9 mm), or severe (>15.0 mm). Twenty-two birds with normal beaks were selected as controls and provided baseline information on normal microanatomy of the chickadee beak and claw.[32] A single bird with a grossly normal beak and an elongated claw was also included as a potentially affected animal. A subset (n = 16 affected, n = 14 controls) of the birds used in this study was held in captivity for up to five months at the University of Alaska Fairbanks (UAF) Biological Research and Diagnostics Facility as part of a

separate investigation prior to post-mortem examination.[33] All birds that died while in captivity (n = 9 affected) were necropsied immediately by UAF Veterinary Services personnel. All other individuals were euthanized with isoflurane using the open-drop method upon capture or at the termination of the captive study and kept cool (~4° C) until necropsy.

This study was completed under guidance of the UAF and the U. S. Geological Survey Alaska Science Center Institutional Animal Care and Use committees (Assurance #07-49, 08-57). Measurements are reported as means ± standard error.

3.3.2 Radiography

High-magnification radiography (Dage XD7500NT, Nordson Corporation, Westlake, OH) was used to examine the underlying bone structure of the beak from a subset of affected birds (n = 18) and controls (n = 14).[32] Images of the head and beak were collected at 20-100x magnification (40kV, 2.8W) using frame averaging (n = 256) with Dage XiDAT software (Nordson Corporation).

3.3.3 Histopathology

All major tissues and organ systems (brain, spinal cord, eyes, heart, lung, kidneys, liver, pancreas, adrenal gland, axial and appendicular bone, skeletal muscle, proventriculus, ventriculus, and intestine) from five affected birds and three controls were examined using light microscopy. To rule out hepatic disease, which has been proposed as a cause of beak hyperkeratosis in other species,[10] liver tissues from an additional 10

affected animals and 10 controls were examined. For detailed histopathology of keratinized tissues, we sampled beak, claw, skin, feather follicles, and epithelial lining of nasal sinuses from 25 affected animals and 19 controls. Tissues were fixed in 10% neutral buffered formalin, processed using an automated tissue processor (Shandon Citadel 2000; GMI Inc., Ramsey, Minnesota), and embedded in paraffin blocks (Reichert-Jung Model 8040 Tissue Embedding Center with vacuum; Reichert Technologies, Buffalo, NY). We used methods described in Van Hemert et al.[32] to decalcify and soften beaks and claws. Beak tissues were trimmed for sectioning along either the mid-sagittal (n = 20 affected, n = 14 controls) or transverse plane (n = 5 affected, n = 5 controls). For transverse sections, the upper and lower beaks in apposition were cut at four reference locations based on the upper beak: immediately proximal to and 1, 3, and 5 mm from the distal edge of the external nares. Skin, feather follicles, and sinuses were included with head and beak sections. The hallux was embedded whole and faced off to achieve a mid-sagittal plane of section. Blocks were cut into 5-μm sections using an American Optical 820 Spencer microtome (American Optical, Buffalo, NY), mounted onto charged glass slides (VWR Superfrost Plus Micros Slide; VWR International, Radnor, PA), and stained with hematoxylin and eosin using standard procedures.[20] Select sections were stained with Masson's trichrome stain for keratin, collagen, and bone (American Master Tech Scientific, Lodi, CA), Periodic acid Schiff stain for carbohydrates (PAS; American Master Tech Scientific), phosphotungstic acid hematoxylin stain for fibrin, collagen, and elastin (American Master Tech Scientific), Gram stain for bacteria (Newcomer Supply, Middleton, WI), Grocott Methenamine

Silver stain for fungi (GMS; Ventana Medical Systems, Tucson, AZ), and Steiner stain for spiral bacteria (Ventana Medical Systems), according to each manufacturer's specifications. External laboratory controls and positive staining within slides were used to confirm the reliability of these stains.

Slides were viewed with a Leica DM4500 microscope (Leica Microsystems GmbH, Wetzlar, Germany) and images were collected with a Leica DFC420 C camera and Leica Application Suite software (Leica Microsystems). Images were prepared using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA).

3.3.4 Transmission electron microscopy

Transmission electron microscopy (TEM) was used to screen for pathogens and to resolve uncertainties in the characterization of lesions that were identified with histopathology in five affected and three unaffected chickadees. Pathogen screening was conducted by negative staining techniques and examination of thin sections from multiple tissues. Detailed ultrastructural examination targeted lesions identified with light microscopy, including areas of hyperkeratosis and the junction between the inner and outer cornified plates. All assessments were made relative to controls.

Fresh feather, skin, liver, kidney, lungs, and intestinal tissues were sampled for virus screening with negative staining. Tissues were placed in 1 ml of doubly distilled water, then homogenized to produce an opalescent suspension and centrifuged at 4000 rpm (Eppendorf, Hamburg, Germany). Supernatant was transferred to airfuge tubes (Belkman Coulter, Brea, CA) and centrifuged at 30 psi using an airfuge (Belkman

Coulter) for 10 min. Supernatant was then removed and the pellet was reconstituted with 10 µl of double-distilled water. We transferred 5 µl of the sample to parafilm and then placed formvar-coated copper grids (Electron Microscopy Sciences, Hatfield, PA) on top of the drop for 10 minutes. Excess liquid was wicked and the grids were stained with 1% phosphotungstic acid (Electron Microscopy Sciences) for 1 min.

For examination of tissue ultrastructure, we fixed beak, nail and skin in 10% buffered formalin immediately after euthanasia. Beak and nail tissues were then decalcified in EDTA for a maximum of 8 weeks. Transverse sections of preselected areas of the beak were collected at two locations: at approximately 2 mm and 5 mm distal of the nares (at the tip of the premaxillary and mandibular bones). Thereafter, samples were post-fixed in 0.166 M cacodylate-buffered, 3% glutaraldehyde with 1% tannic acid solution (Electron Microscopy Sciences), followed by a second postfixation treatment in 1% osmium tetroxide (Electron Microscopy Sciences). We then dehydrated 1.0-mm^3 tissue blocks using a graded series of ethyl alcohol and embedded these in Embed (Electron Microscopy Sciences). Embedded samples were trimmed and sectioned on a Leica UC6 Ultramicrotome (Leica Microsystems). Thin sections (60–90 nm) were collected on 100-mesh copper grids (Electron Microscopy Sciences), then grids were stained with 5% uranyl acetate for 20 min and Satos' lead citrate for 6 min.

We examined samples with a JEOL 1200 EX II transmission electron microscope (JEOL LTD, Tokyo, Japan) and obtained images using a Veleta 2K x2K camera with iTEM software (Olympus SIS, Munster, Germany).

3.4 RESULTS

3.4.1 Gross pathology

Affected birds exhibited elongation of the upper beak, lower beak, or both, with varying degrees of lateral deviation, crossing, and gap between mandibles. Deformities ranged from mild overgrowth to severe crossing and elongation (Fig. 3.1). At necropsy, beak measurements of affected birds (n = 30) averaged 12.8 ± 1.0 mm (6.8–25.0 mm) from nares to tip and 9.5 ± 0.67 mm (6.3–18.9) mm along the gonys. For control beaks (n = 22), the mean nares-to-tip length was 7.3 ± 0.1 mm (6.0–7.8 mm) and mean gonys length was 6.6 ± 0.1 mm (6.0–7.9 mm). In affected birds, the surface of the beak was typically rough and irregular, with raised lateral ridges and discoloration (pale or white compared to normal black pigmentation). The superficial layers of the hard-cornified beak epidermis sometimes also appeared thickened or exhibited flaking or peeling. Upon histologic sectioning, hard-cornified beak tissues of affected birds were noticeably more brittle and more easily fractured than those of unaffected birds.

Among 30 birds with beak deformities, 5 (17 ± 7%) exhibited one or more elongated claws and 3 (10 ± 6%) exhibited other claw abnormalities, including thickening, raised lateral ridges, and peeling of superficial layers. In addition to the single bird that was captured with an apparently normal beak and severely elongated middle claw, macroscopic claw lesions were identified in 2 of 25 control birds. One (5 ± 4%) displayed a slightly elongated hallux and another (5 ± 4%) showed lateral ridges in

several claws. Both of these birds had grossly normal claws at capture and developed lesions while in captivity.

Mild to severe alopecia, xerosis, scaling, and/or exudation were present on the head, face, lores, or abdomen in 25 of 30 (83 ± 7%) affected birds; lesions were most severe in birds previously housed in captivity. Of the 22 control birds, 3 (14 ± 7%) that had been previously housed in captivity exhibited mild skin lesions.

3.4.2 Radiography

The underlying bones of the beak appeared to be of approximately normal length (Figs. 3.1b, g, l), but bone deformation at the tip of the premaxilla and/or mandible was evident in 15 of the 18 (83 ± 9%) affected birds that were examined by high-magnification radiography. Deviation from the frontal plane toward superficial aspects of the beak resulted in recurved and decurved appearance of the premaxilla and mandible, respectively (Figs. 3.1c,h,m). In most of these cases, the apex of the bone also appeared indistinct or irregular, which contrasted with the clearly demarcated, consistent margins observed in birds with normal beaks. Severity of bone lesions was typically proportional to the amount of beak overgrowth (Fig. 3.1).

3.4.3 Histopathology

Hyperkeratosis occurred in the cornified plate of nearly all abnormal beaks (Table 3.1). Severity of hyperkeratosis varied widely, ranging from slight thickening of the cornified epidermis to thickening and elongation to more than triple the beak's normal

size (Figs. 3.1d,i,n). Other abnormal features, including hyperplasia and dermal and osseous lesions, typically correlated with the relative amount of beak overgrowth (Figs. 3.1e,j,o).

The *stratum corneum* in affected beaks appeared disorganized under polarized light microscopy, demonstrated by attenuation and irregular, swirling, and scalloped patterns of birefringence (Fig. 3.2b) compared to the typical linear arrangement observed in normal beaks (Fig. 3.2a).[32] Individual corneocytes were disarrayed along the juncture between the external and buccal plates in birds with moderate to severe beak overgrowth. There was increased exfoliation of the cornified laminae along external surfaces of the beak in affected birds. Microbial pathogens were occasionally observed in these fissures; two ($8.0 \pm 5.4\%$; $n = 25$) specimens exhibited isolated pockets of Gram-positive cocci and three ($12 \pm 7\%$; $n = 25$) other specimens displayed PAS-positive fungi in the superficial layers of the upper or lower beak.

Cells that resembled those from the *stratum transitivum*, characterized by retention of the nucleus, increased eosinophilia, and polyhedral rather than flattened morphology[32] (Figs. 3.3a–c), occurred frequently in the *stratum corneum* of affected beaks. These transitional cells were often concentrated near the apex of the dermis, along the margins of the juncture between the inner and outer cornified plates, and, in some cases, sequestered in linear formations parallel to the germinal epithelium (Figs. 3.1n–o, 3.3a). Globular, lacuna-like lesions containing amorphous, eosinophilic material were also common in the *stratum corneum* of abnormal beaks (Fig. 3.4a). These lacunae were

diffusely distributed along the cornified laminae and occupied spaces varying from the size of a single corneocyte (~10 μm) to more than 150 μm in length (Figs. 3.4e–f).

A core-like intrusion that originated near the apex of the dermis and continued along the junctional line of the external and buccal plates of the *stratum corneum*, often persisting to the tip of the beak, was common in affected birds (Fig. 3.4a), especially in those with moderate to severe beak overgrowth. This characteristic lesion consisted of a columnar aggregate of tightly clustered nuclear remnants of red and white blood cells and amorphous, eosinophilic debris, sometimes interspersed with eosinophilic globules (Figs. 3.4a–c). PAS, Gram, GMS, and Steiner staining of this material was negative and no other evidence of microbial pathogens was detected in these areas. Similar lesions were not observed in controls.

Mild to moderate hyperplasia and occasional mild dysplasia was present in the germinal epithelium of the beak in affected animals (Figs. 3.3a,d). In some focal areas, typically near the apex of the dermis, the basal layer increased from a single band of keratinocytes to a disorganized stratum several cells thick. In many cases, these basal cells were also hypertrophic and exhibited irregular, prismatic morphology compared to the normal cuboidal shapes observed in controls.[32] Seven of 25 (28 ± 9%) affected animals and no controls presented with intranuclear, eosinophilic inclusions, sometimes accompanied by intracytoplasmic, eosinophilic inclusions, in the germinative layer of the beak. These inclusions were typically not widespread and were difficult to locate. Material recovered from paraffin blocks was not suitable for electron microscopy and

additional study will be required to determine the nature of the inclusions and if viral particles are present.

Lesions consistently observed in the dermis of affected beaks included neovascularization, edema, fibrosis, hyperpigmentation, and pigmentary incontinence (Figs. 3.1e,j,o, 3.3e). In some cases mild inflammation, perineuritis, axonal degeneration and demyelination, and denervation were also present. Lesions typically occurred near the apex of the dermis and were most notable in severely affected birds. Birds with normal beaks did not exhibit any consistent dermal lesions.

Osseous lesions, including new bone formation, osteomyelitis, and myelofibrosis, occurred in the underlying bones of the beak in most affected birds (Figs. 3.1e,j,o). These typically occurred near the tip of the premaxilla or the mandible. Affected beaks exhibited increased scalloping along the external margins of lamellar bone, disconnected fragments of highly remodeled tissue, osteoclastic resorption, hypercellularity, and localized proliferation of woven bone (Fig. 3.3e). These features contrasted with the more quiescent, continuous lamellar bone structure typical of controls.[32] In affected beaks, marrow spaces often contained necrotic debris, fibrosis, and inflammatory cells. Typically, the severity of lesions observed in bone was positively correlated with the gross overgrowth of the beak (Table 3.1, Fig. 3.1).

Among affected birds ($n = 25$), the epithelial lining of the nasal sinuses often demonstrated mild to moderate hyperkeratosis ($40 \pm 10\%$) and occasional mild hyperplasia ($16 \pm 7\%$), typically in association with bacterial or fungal pathogens (Fig. 3.5e). These features were more common among birds previously housed in captivity (*n*

= 8) but also occurred in some wild-caught birds (n = 2). In 2 cases (1 captive, 1 wild), mild lymphoplasmatic infiltration was also present. Four birds with normal beaks (21 ± 9%; n = 19), all of which had been previously housed in captivity, exhibited bacterial infection with mild hyperkeratosis in the sinuses.

Mild hyperkeratosis was observed in the claws of most affected birds (68 ± 9%; n = 25) and in several controls (21 ± 9%; n = 19). A high proportion of birds with abnormal beaks (64 ± 10%; n = 25) and more than one third of controls (37 ± 11%; n = 19) also exhibited core-like intrusions of nuclear remnants and amorphous debris similar to those described in beaks (Figs. 3.5a–b). These lesions originated near the dermal apex and typically continued along the juncture of the dorsal and ventral plates of the claw, often extending to its distal tip. The single individual that was identified at the outset of the study as potentially affected (with a grossly normal beak and elongated claw) exhibited no lesions in the beak but hyperkeratosis, core-like intrusions, and PAS-positive yeast-like organisms in the cornified tissue of both the affected middle claw and the hallux. No other microorganisms were detected in claw tissues, but intranuclear, eosinophilic inclusions similar to those found in the beak in the germinative layer of the claw occurred in several affected birds. Lesions in the *stratum corneum* of the claw demonstrated nearly identical staining and refractive properties to those in the beak. However, claws were more difficult to process for histology than beaks and the quality of these slides was not adequate to consistently evaluate the germinative layer, dermis, or bone.

Lesions in skin and feather follicles were observed in all affected birds ($n = 25$) and in several controls ($32 \pm 11\%$; $n = 19$). These lesions were characterized by hyperkeratosis, occasional hyperplasia, and the presence of microbial pathogens (Figs. 3.5c–d). Similar to macroscopic observations, microscopic lesions were most remarkable in birds with severe beak overgrowth, particularly those that had been previously housed in captivity. Mild and moderate cases were restricted to the superficial layers of the epidermis and did not elicit a visible dermal response. Severe cases included inflammation in the dermis, with lymphoplasmatic infiltration and mild to moderate edema. Lesions in birds with normal beaks were mild and occurred only among the subset of birds previously housed in captivity. Lesions in affected free-ranging birds were mild or moderate, whereas lesions in affected birds previously housed in captivity ranged from mild to severe. Skin samples from two severe captive cases were submitted to the Washington Animal Disease Diagnostic Laboratory. Microbial analysis demonstrated mixed bacterial growth, dominated by coagulase-negative *Staphylococcus* species, and presence of *Penicillium* species.

Lesions were also present in several other tissues, although these occurred inconsistently. One affected specimen exhibited a focal, moderate area of necrosis with histiocytic infiltration in skeletal muscle of the breast and mild, multifocal lymphocytic portal hepatitis. One affected and one unaffected specimen each had multifocal, moderate areas of necrosis with histiocytic infiltration in the myocardium and a small area of coagulative necrosis of the mucosa in the proventriculus. A lipoma removed from the subcutis of the furcular hollow of one severely affected bird demonstrated well-

differentiated adipocytes encapsulated within multiple lobules. All other tissues appeared unremarkable.

3.4.4 Transmission electron microscopy

Electron microscopy demonstrated morphological changes and increased retention of chromatin in corneocytes of affected beaks relative to controls. These cells typically appeared more swollen, showed highly indented cell membranes, and contained more chromatin and nuclear debris than normal corneocytes (Figs. 3.3f–g). However, as observed with light microscopy, TEM confirmed that these lesions affected only a portion of the cells of the *stratum corneum* in abnormal beaks. In some regions, particularly near the base of the beak, the ultrastructure of the corneocytes was indistinguishable between affected animals and controls. In addition, the individual cell components of corneocytes from affected animals were not obviously abnormal and desmosomes appeared intact. The appearance of ß-keratin bundles and melanin granules contained within cells did not obviously differ between affected beaks and controls upon qualitative examination. Ultrastructural changes observed in the *stratum germinativum*, dermis, and bone corresponded with histologic lesions (Figs. 3.3h–i).

The lacuna-like lesions observed along laminae of the *stratum corneum* contained disorganized, variably dense packets of keratin filaments that were consistent with degenerated or necrotic corneocytes (Fig. 3.4g). These formed small to large granular and finely filamentous aggregations that appeared to correspond with the eosinophilic globules visible with light microscopy. The characteristic core-like intrusions that

occurred along the juncture between the external and buccal plates of the *stratum corneum* consisted of free-floating erythrocytes, inflammatory cells, keratinocytes, and corneocyte debris (Fig. 3.4d). These cells and associated debris were not confined within an endothelium and were in direct contact with adjacent corneocytes of the *stratum corneum*. Increased degeneration of corneocytes was also apparent along the margins of this juncture in affected beaks. Ultrastructural examination did not reveal any evidence of microorganisms in these areas, confirming results from special stains and light microscopy. Abnormalities of the nucleus, including cytoplasmic invaginations composed of dense packets of fine filaments, were occasionally observed in the *stratum germinativum*. Additional study is required to determine if these lesions correspond to the eosinophilic, intranuclear inclusions that were visible with light microscopy.

Electron microscopy did not reveal consistent evidence of viruses in affected animals. Although viral particles were detected in several birds, these differed among individuals in both location and appearance. In thin sections, intracytoplasmic virus-like particles 80–100 nm in diameter were present in mesenchymal cells of the dermis of the upper beak of one moderately affected bird and virus-like particles ~100 nm in diameter were free in the intercellular space in the germinal epithelium of the lower beak of one severely affected bird. Negative staining demonstrated 90–100 nm non-enveloped icosahedral particles of the Family Adenoviridae in the liver of one mildly affected bird and in the kidney of another mildly affected bird. Viral particles were not detected in any other tissues of affected birds or controls.

3.5 DISCUSSION

This study was prompted by a recent outbreak of beak deformities in thousands of Black-capped Chickadees and other wild birds in Alaska. Reports of beak abnormalities are not uncommon in the published literature, but these are typically limited to a single gross observation or clinical case without corresponding in-depth pathologic examination and description. Detailed study of the beak and other keratinized tissues has been conducted in association with certain toxicoses,[23] nutritional disorders,[22,36] and viral diseases,[5,26,28] which typically exhibit distinctive features. The gross, microscopic, and ultrastructural lesions that we observed in affected Black-capped Chickadees do not closely resemble these or any other known avian diseases, suggesting a novel disorder in wild birds.

The microscopic changes that we observed in affected chickadees are consistent with rapid production of keratin resulting in overgrowth of the rhamphotheca. The most characteristic features of this disorder occurred in the beak epidermis and included hyperplasia and hyperkeratosis. We also observed discrete, multifocal clusters of corneocytes with altered morphology and increased retention of chromatin. These cells appeared similar to those from the *stratum transitivum* and may have been the product of rapid differentiation associated with accelerated epidermal growth. This explanation concurs with results from a previous study in which we documented increased rates of beak growth in chickadees with beak overgrowth and deformity.[33] The location of these less mature cells, typically near the tip of the dermis and in distal regions of the *stratum*

corneum, also corresponds with the dominant longitudinal growth of the beak epidermis.[21,29,33]

Other lesions in affected beaks suggested disruption of the integrity of the cornified plate of the beak. We consistently encountered increased brittleness and fracturing during processing and observed disorganization of the cornified laminae with polarized light. Microscopically, affected beaks exhibited degeneration of corneocytes, resulting in lacuna-like lesions throughout the *stratum corneum* and altered structure along the margins of the juncture between the inner and outer plates of the beak. However, intracellular components of individual corneocytes, including ß-keratin bundles, did not appear to differ ultrastructurally between normal and affected beaks.

The core-like intrusions that were common in birds with beak deformities consisted of red and white blood cells and corneocyte debris. These lesions occurred in a restricted region of the *stratum corneum* where inner and outer horn layers merge. In a normal beak, columns of transitional cells form a structurally significant junction at this site,[18,32] which presents a break in the otherwise continuous barrier formed by laminae of the hard-cornified epidermis. Thus, this junction may provide a natural channel through which free-floating cells and necrotic debris can be routed. Neovascularization at the tip of the dermis was a common finding in affected birds and it is possible that hemorrhage due to trauma or chronic strain associated with overgrowth of the beak could partially explain the presence of erythrocytes in these areas. Chronic strain to the beak tip may also contribute to the infiltration of inflammatory cells that we observed in the dermis and along the junctional line of the beak. In moderately to severely affected birds, the core-

like intrusions of necrotic material sometimes continued all the way to the distal tip of the beak. Despite this potential breach of the beak integument, we did not detect any pathogens within these areas.

In addition to abnormalities in the epidermis, we also observed significant bone remodeling and various osseous and dermal lesions, particularly in birds with moderate to severe beak overgrowth. We reasoned that this pattern could be explained by one of two competing hypotheses: 1) osseous and dermal lesions are associated with the primary pathogenesis of beak deformities and may subsequently induce abnormal growth of the beak epidermis or, 2) osseous and dermal lesions are a secondary consequence of trauma associated with a grossly elongated beak. Histologic and radiographic examination of cases across a wide range of severity and with known duration for some individuals allowed us to evaluate these possibilities. Due to the identification of this disorder via beak overgrowth, which typically requires at least several weeks to be detectable macroscopically,[33] we considered all lesions in affected birds to be chronic. However, in several birds with slight deformities, we observed no obvious dermal or osseous lesions, suggesting that epidermal lesions may precede changes in other tissues of the beak. Similarly, for birds that had relatively recent onset of deformities (<6 months), we observed no or only mild lesions in the bone and dermis. Generally, the most severe osseous and dermal lesions occurred in birds with the most pronounced beak overgrowth. In addition, the recurved and decurved appearance of the premaxilla and mandible, respectively, correlates with the direction of torque that would be exerted on rhamphothecal tips in grossly elongated beaks. It is therefore plausible that bone

remodeling may occur in response to increased mechanical strain associated with such forces. Based on these observations, we suspect that osseous and vascular changes are secondary consequences rather than primary lesions of the disorder. Although logistically challenging due to the difficulty of early detection in wild birds, repeated radiographic examination from the point of onset to development of severe lesions combined with targeted histopathology to determine the nature of the lesion would be useful for evaluating this pathologic sequelae. It is also likely that lesions in all beak tissues (epidermis, dermis, bone) may be further exacerbated by amplification of the inflammatory response associated with repeated, chronic stress to the elongated beak.

We observed some evidence that multiple keratinized tissues are affected by this disorder. Claws, which share many biochemical and structural properties with beaks,[2,6,13] exhibited similar pathology. Although we only observed gross overgrowth of the claw in a small number of affected individuals, we frequently detected mild hyperkeratosis and core-like intrusions of red and white blood cells and corneocyte debris similar to those found in the beak upon microscopic examination. The occurrence of nearly identical lesions in the claw suggests that both of these tissues are affected similarly, despite the more conspicuous presentation in the beak. We did not explicitly measure claw length so it is possible that overgrowth occurred more frequently than we detected macroscopically. In addition, because claws are thinner and more fragile than beaks, they may be more likely to break when they become elongated, potentially masking the effects of hyperkeratosis. We were also surprised to find a relatively high rate of core like-intrusions in claws of birds with apparently normal beaks. In most of these cases, we did

not observe any macroscopic lesions, although this may have been due in part to the difficulty of detecting overgrowth in claws as described above. The presence of these lesions in claws of birds with normal beaks suggests that the disorder may manifest first in either the beak or the claw, and may not immediately result in changes that are grossly detectable. Given the resemblance between the lesions in beaks and claws, this explanation seems likely. However, it is also possible that the core-like lesions that we observed in the claw are not unique to this disorder and could be explained by other etiologies. For example, if hemorrhage is an important factor for producing such lesions, breakage of claws due to normal wear and tear could result in similar presentation.

Birds with beak deformities exhibited much higher rates of hyperkeratosis and hyperplasia in skin, feather follicles, and sinus epithelium than birds with normal beaks. However, we observed microbial pathogens in nearly all of these cases, suggesting that epidermal lesions are likely to be secondary consequences of opportunistic infection. Bacterial and fungal infections in skin, feather follicles, and sinuses of birds with beak deformities could be related to proximate causes such as compromised preening ability, poor nutritional status, or reduced immune function,[3] which may be directly or indirectly related to this disorder. Although not explicitly assessed in this study, it is also possible that the integrity of skin integument may be compromised, thus reducing the skin's ability to serve as a natural barrier. The α-keratins in skin, feathers, and sinus epithelium are of a different structure than the ß-keratins of the beak and claws,[13] but processes of keratinization are similar and may be subject to some of the same controls.[12] In general, epidermal lesions were more severe in affected birds housed in captivity than birds

sampled directly from the wild. Captive conditions, including warmer ambient temperatures, may have promoted microbial growth, and may partially explain the observed differences in severity. However, the presence of similar lesions in both groups suggests that they are not merely a product of captivity and may be an important secondary consequence of this disorder. Given the harsh environmental conditions of winter in Alaska, epidermal infection would significantly compromise a bird's ability to thermoregulate and may contribute to mortalities, thus reducing the likelihood that we would encounter free-ranging birds with severe lesions.

We did not find consistent evidence of bacterial, fungal, or viral pathogens that could explain the etiology of this disorder. These results correspond with previous diagnostic testing of affected chickadees.[8] However, further research is needed to evaluate whether intranuclear inclusions identified in the germinative layer of the beak and claw in some affected birds could be due to the presence of a virus or, alternatively, may be non-pathogenic cytoplasmic invaginations. Some viral diseases, including psittacine beak and feather disease and avian pox, have been reported to cause hyperkeratosis of the beak and other keratinized tissues, but these are typically accompanied by characteristic lesions in other tissues.[5,25,28] The gross and microscopic presentation of non-keratinized tissues in chickadees was generally unremarkable and no viruses were identified in negative staining of organs known to host circoviruses, pox viruses, and polyoma viruses in other species.[5] The inclusions we observed with light microscopy differed in appearance from viral inclusions that are typical of circovirus or avian pox virus, but more closely resembled polyoma, herpes, and adenoviruses.[5]

Our findings are not consistent with any known avian diseases and contradict most differential diagnoses. Overgrowth of the rhamphotheca is sometimes reported in captive birds, but such lesions are usually attributed to lack of wear,[1] rather than to accelerated growth, which has been demonstrated in association with beak deformities in Black-capped Chickadees.[33] Metabolic disorders, such as those related to deficiencies of Vitamin A, zinc, or biotin, may also be responsible for beak overgrowth and abnormalities in other keratinized tissues,[15,17] but chickadees with beak deformities did not display lesions typical of these or other reported nutritional problems. In addition, previous micronutrient analyses did not reveal differences between affected and unaffected birds.[9] Beak abnormalities previously attributed to toxic etiologies in other avian species, including exposure to elevated levels of selenium or organochlorines,[7,19,24] exhibit very different gross and microscopic lesions than what we observed in this study. We found no evidence of lesions in the liver or other organs commonly affected by some toxicants[27] and preliminary toxicological analyses did not identify abnormally high levels of metals or organochlorines in affected birds.[9]

The term "avian keratin disorder" has been used to describe the suite of macroscopic features, including beak overgrowth, that are observed in keratinized tissues of affected birds. Microscopic and ultrastructural examination allowed us to confirm and characterize lesions in beak, claw, skin, feather, and sinuses, however, we were unable to determine whether these corresponded with a primary keratin disorder. Biochemical analysis and immunohistochemistry of beak and claw tissues would provide insights about possible abnormalities in keratin expression and help to clarify the associated

terminology. Other diagnostic testing, including investigation of the inclusions observed in this study, is necessary to identify likely etiological agents. Dioxins and dioxin-like compounds have been associated with keratin disorders in other taxa[14,16,34,35] and also warrant additional study. Epidemiological analyses may provide important insights about whether an infectious mode of transmission is likely. Recent patterns of emergence suggest rapid spread across a large geographic area and highlight the potential for this emerging disease to have significant effects on wild bird populations.

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3.7 LITERATURE CITED

1. Arnall L: Conditions of the beak and claw in the Budgerigar. Journal of Small Animal Practice **6:** 135-144, 1965.
2. Brush AH: Patterns in the amino acid compositions of avian epidermal proteins. Auk **97:** 742-753, 1980.
3. Cooper JE, Harrison GJ: Dermatology. *In*: Avian Medicine: Principles and Application, eds. Ritchie BW, Harrison GJ, Harrison LR, pp. 607-639. Wingers Publishing, Lake Worth, FL, 1994.
4. D'Alba L, Van Hemert C, Handel CM, Shawkey MD: A natural experiment on the condition-dependence of achromatic plumage reflectance in Black-capped Chickadees. PLoS ONE **6:** e25877, 2011.
5. Fletcher OJ, Abdul-Aziz T: Avian Histopathology, 3rd ed. p. 438. American Association of Avian Pathologists, Jacksonville, Fla., 2008.
6. Frenkel MJ, Gillespie JM: The proteins of the keratin component of bird's beaks. Australian Journal of Biological Sciences **29:** 467-479, 1976.
7. Gilbertson M, Kubiak T, Ludwig J, Fox G: Great Lakes embryo mortality, edema, and deformities syndrome (GLEMEDS) in colonial fish-eating birds: similarity to chick-edema disease. Journal of Toxicology and Environmental Health **33:** 455-520, 1991.

8. Handel CM, Pajot LM, Matsuoka SM, Hemert CV, Terenzi J, Talbot SL, Mulcahy DM, Meteyer CU, Trust KA: Epizootic of beak deformities among wild birds in Alaska: an emerging disease in North America? Auk **127:** 882-898, 2010.

9. Handel CM, Pajot LM, Matsuoka SM, Trust KA, Stotts JM, Terenzi J, Talbot SL: Potential role of environmental contaminants in the pathology of beak deformities among Black-capped Chickadees in south-central Alaska. USGS Alaska Science Center, Anchorage, Alaska, 2006.

10. Harrison GJ: Disorders of the integument. *In*: Clinical Avian Medicine and Surgery, eds. Harrison GJ, Harrison LR, pp. 397-407. W. B. Saunders Co., Philadelphia, Pennsylvania., 1986.

11. Harrison T: Beak deformities of garden birds. British Birds **104:** 538-541, 2011.

12. Homberger DG: The case of the cockatoo bill, horse hoof, rhinoceros horn, whale baleen, and turkey beard: the integument as a model system to explore the concepts of homology and non-homology. *In*: Vertebrate Functional Morphology: Horizon of Research in the 21st Century, eds. Dutta HMDatta Munshi JS, pp. 317-343. Science Publishers, Enfield, New Hampshire, 2001.

13. Homberger DG, Brush AH: Functional-morphological and biochemical correlations of the keratinized structures in the African Grey Parrot, *Psittacus erithacus* (Aves). Zoomorphology **106:** 103-114, 1986.

14. Jackson T, Halbert F: A toxic syndrome associated with the feeding of polybrominated biphenyl-contaminated protein concentrate to dairy cattle. Journal of the American Veterinary Medical Association **165:** 437-439, 1974.

15. Keymer IF, Samour J: Disorders of the digestive system. *In*: Avian Medicine, ed. Samour J, 2nd ed., pp. 281-302. Elsevier, New York, 2008.

16. Kimbrough RD: Skin lesions in animals and humans: a brief overview. Banbury Report **18:** 357-363, 1984.

17. Klasing KC: Comparative Avian Nutrition Cab International, New York, 1998.

18. Lucas AM, Stettenheim PR: Avian Anatomy: Integument. *In*: Agriculture Handbook 362. U.S. Department of Agriculture, Washington, D. C., 1972.

19. Ludwig JP, Kurita-Matsuba H, Auman HJ, Ludwig ME, Summer CL, Giesy JP, Tillitt DE, Jones PD: Deformities, PCBs, and TCDD-equivalents in Double-crested Cormorants (*Phalacrocorax auritus*) and Caspian Terns (*Hydroprogne caspia*) of the upper Great Lakes 1986-1991: testing a cause-effect hypothesis. Journal of Great Lakes Research **22:** 172-197, 1996.

20. Luna G: AFIP Manual of Histologic Staining Techniques, 3rd ed., p. 258. McGraw-Hill Publications, New York, 1968.

21. Menzel R, Lüdicke M: Funktionell-anatomische und autoradiographische Untersuchungen am Schnabelhorn von Papageien (Psittaci). Zoologische Jahrbücher, Abteilung für Anatomie und Ontogenie der Tiere **93:** 175-218, 1974.

22. Monroe AD, Latimer KS, Pesti GM, Bakalli RI: Pathology and histology of dietary tryptophan deficiency in broiler chicks. Avian Diseases **47:** 1393-1398, 2003.

23. O'Toole D, Raisbeck MF: Experimentally induced selenosis of adult Mallard ducks: clinical signs, lesions, and toxicology. Veterinary Pathology **34:** 330-340, 1997.

24. Ohlendorf HM, Hoffman DJ, Saiki MK, Aldrich TW: Embryonic mortality and abnormalities of aquatic birds: apparent impacts of selenium from irrigation drainwater. Science of the Total Environment **52:** 49-63, 1986.

25. Olsen GH: Oral biology and beak disorders of birds. Veterinary Clinics of North America Exotic Animal Practice **6:** 505-521, 2003.

26. Pass DA, Perry RA: The pathology of psittacine beak and feather disease. Australian Veterinary Journal **61:** 69-74, 1984.

27. Poppenga RH: Avian toxicology. *In*: Veterinary toxicology: Basic and Clinical Principles, ed. Gupta RC, pp. 663-688. Elsevier, New York, 2007.

28. Schmidt RE, Reavill DR, Phalen DN: Pathology of Pet and Aviary Birds, 1st ed. p. 234. Iowa State Press, Ames, Iowa, 2003.

29. Seki Y, Bodde SG, Meyers MA: Toucan and hornbill beaks: a comparative study. Acta Biomaterialia **6:** 331-343, 2010.

30. Tully TN, Jr., Lawton MPC, Dorrestein GM: Handbook of Avian Medicine, 2nd ed. Butterworth-Heinemann, Elsevier Science Ltd., New York, 2000.

31. Van Hemert C, Handel CM: Beak deformities in Northwestern Crows: evidence of a multispecies epizootic. Auk **127:** 746-751, 2010.

32. Van Hemert C, Handel CM, Blake JE, Swor RM, O'Hara TM: Microanatomy of passerine hard-cornified tissues: beak and claw structure of the Black-capped Chickadee (*Poecile atricapillus*). Journal of Morphology: **273:**226-240, 2012.

33. Van Hemert C, Handel CM, O'Hara TM: Evidence of accelerated beak growth associated with avian keratin disorder in Black-capped Chickadees (*Poecile atricapillus*). Journal of Wildlife Diseases: in press.

34. Vos JG, Beems RB: Dermal toxicity studies of technical polychlorinated biphenyls and fractions thereof in rabbits. Toxicology and Applied Pharmacology **19:** 617-633, 1971.

35. White SS, Birnbaum LS: An overview of the effects of dioxins and dioxin-like compounds on vertebrates, as documented in human and ecological epidemiology. Journal of Environmental Science and Health, Part C: Environmental Carcinogenesis and Ecotoxicology Reviews **27:** 197-211, 2009.

36. Wight PAL, Dewar WA: The histopathology of zinc deficiency in ducks. The Journal of Pathology **120:** 183-191, 1976.

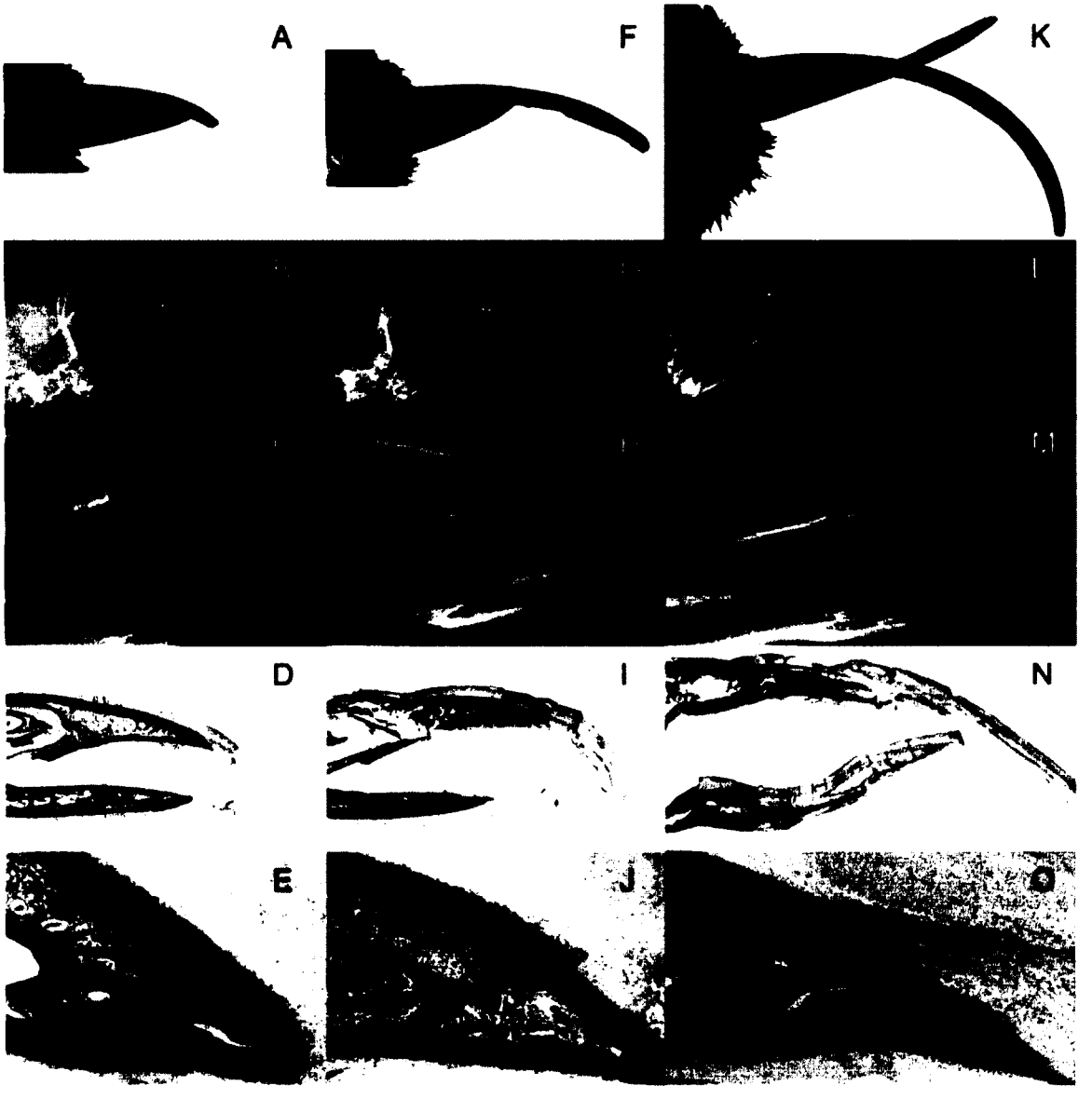

Figure 3.1 Examples of gross, radiographic, and microscopic (hematoxylin and eosin) presentation of beak overgrowth in the head and beak of Black-capped Chickadees. Images depict mild (A–E), moderate (F–J), and severe (K–O) cases. Characteristic lesions include gross overgrowth of the beak, hyperkeratosis, and epidermal hyperplasia. In moderate and severe cases, bone remodeling, myelofibrosis, osteomyelitis, neovascularization, fibrosis, and hyperpigmentation were also present. Severity of lesions was typically proportional to the relative amount of beak overgrowth.

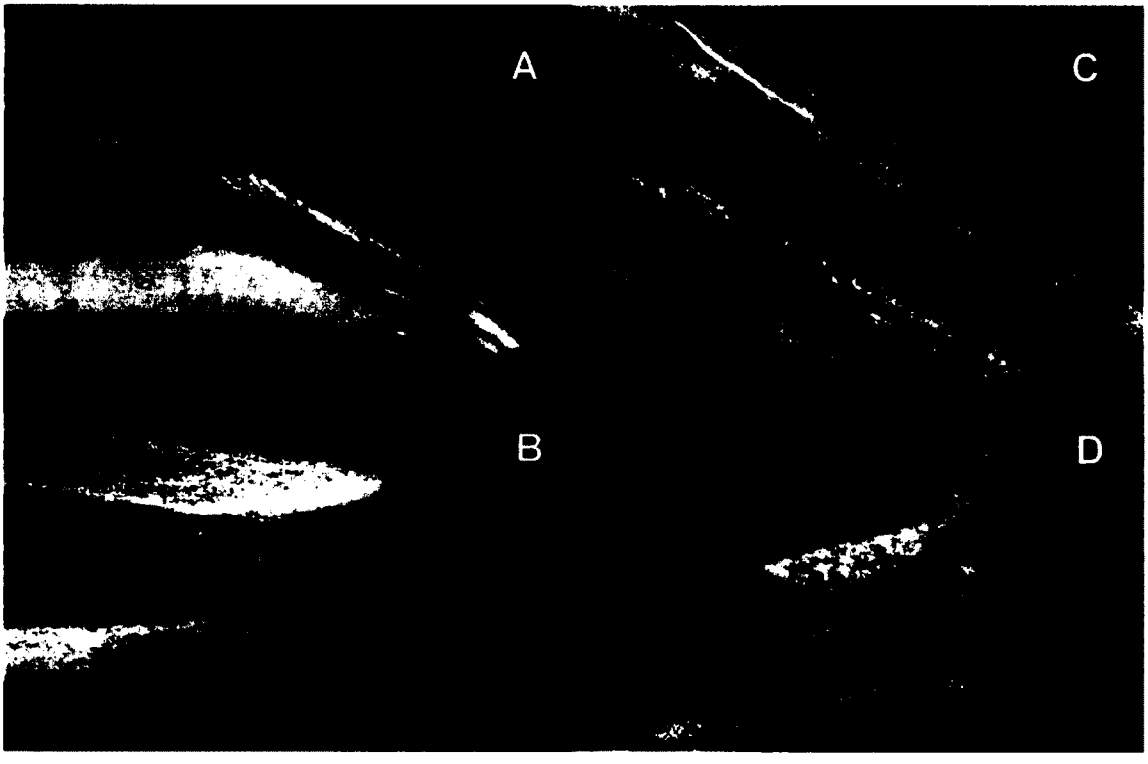

Figure 3.2 Polarized light micrographs (hematoxylin and eosin) of Black-capped Chickadee beak. A normal upper (A) and lower (B) beak shows typical linear patterns of birefringence. Disorganization of the *stratum corneum*, evidenced by attenuation and irregular swirling and scalloping, is shown in the upper (C) and lower (D) beak of a bird with moderate beak overgrowth.

Figure 3.3 Light (A–E, hematoxylin and eosin) and electron micrographs (F–I) of a Black-capped Chickadee with severe beak overgrowth. Insets in A correspond to, from left to right, the *stratum corneum* (B, F), areas of the *stratum corneum* with retention of nuclear debris (C, G), the *stratum germinativum* (D, H), and the interface between the bone and dermis (E, I). Lesions evident in the *stratum corneum* include increased indentation of cell membranes (F) and altered morphology and incomplete differentiation of corneocytes in some regions (C, G). The *stratum germinativum* displays hyperplasia and disorganization of basal cells (D, H). Bone tissue exhibits increased cellularity (E) and active osteoblasts (I). Connective tissue shows variable fibrosis and inflammation (E, I).

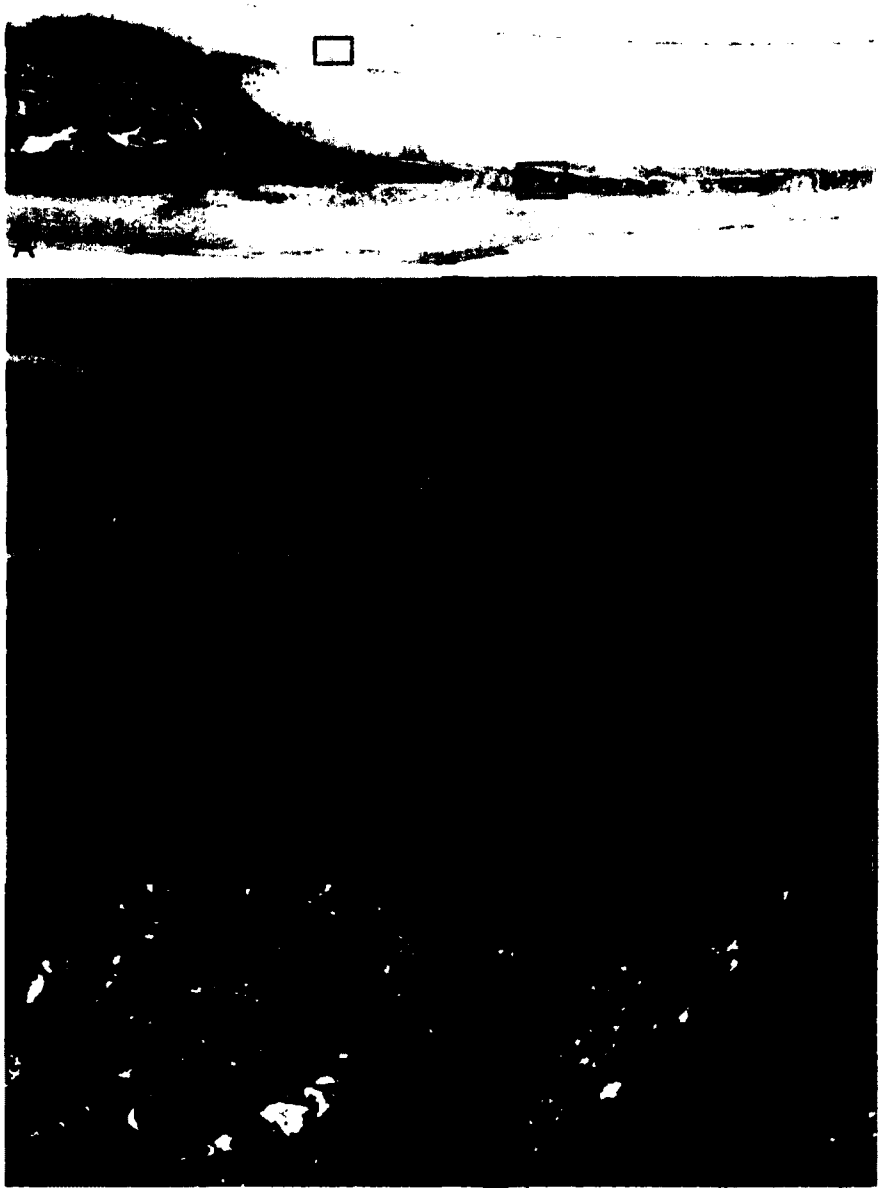

Figure 3.4 Lower beak of Black-capped Chickadee with severe overgrowth showing two common lesions. Insets (A) highlight a core-like intrusion (lower box) and eosinophilic, lacuna-like globules (upper box) in the *stratum corneum* (hematoxylin and eosin). Core-like intrusions along the junctional line of the inner and outer cornified plate are composed of cellular debris from red and white blood cells and corneocytes, shown with hematoxylin and eosin (B) and toluidine blue (C). Electron microscopy demonstrates amorphous globules of degenerating corneocytes in these areas (D). Lacuna-like lesions in the *stratum corneum* (E, hematoxylin and eosin; F, toluidine blue) are formed by degenerating corneocytes, as evident with electron microscopy (G).

Figure 3.5 Lesions in other keratinized tissues in Black-capped Chickadees. Claw showing hyperkeratosis, hyperplasia, and a core-like intrusion of highly nucleated cellular debris (A, B). Hyperkeratosis and hyperplasia of the skin associated with fungal (C) and bacterial (D) pathogens. Nasal sinus with severe hyperkeratosis accompanied by bacterial infection (E). Hematoxylin and eosin

CHAPTER 4. STABLE ISOTOPES IDENTIFY DIETARY CHANGES ASSOCIATED WITH BEAK DEFORMITIES IN BLACK-CAPPED CHICKADEES (*POECILE ATRICAPILLUS*)[1]

4.1 ABSTRACT

A large number of beak deformities of unknown etiology have recently been reported in black-capped chickadees (*Poecile atricapillus*) and other resident avian species in Alaska. In this study we investigated the potential association between diet and beak deformities. We analyzed carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes in whole blood of black-capped chickadees captured at three semi-urban sites in south-central Alaska. For dietary analysis, we included natural foods (arthropods, seeds, and berries) and anthropogenic items commonly provided in bird feeders (sunflower seeds, peanut butter, suet). Blood samples from chickadees with beak deformities exhibited lower $\delta^{15}N$ values and more variable $\delta^{13}C$ values than birds with normal beaks. Isotopic values of blood also differed by location for both carbon and nitrogen, but we did not detect a difference in natural dietary items across the three sites. Contributions of individual diet items differed between birds with and without beak deformities, a pattern which likely reflected reduced function of the beak. Affected birds generally consumed fewer arthropods and sunflower seeds and more peanut butter and natural seeds and berries. Although some individuals with beak deformities relied heavily on feeder foods, we did not find evidence of an anthropogenic food source shared by all affected birds. In

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addition, dietary differences were most pronounced for moderately to severely affected birds, suggesting that these differences are more likely to be a consequence than a cause of deformities.

4.2 INTRODUCTION

We recently documented an epizootic of beak deformities in black-capped chickadees (*Poecile atricapillus*) and other resident bird species in Alaska (Handel et al. 2010; Van Hemert and Handel 2010). Despite ongoing research on this problem, which has been termed "avian keratin disorder," its etiology is currently unknown. Suspected and known causes of beak deformities in birds include infectious agents, parasites, genetic abnormalities, exposure to environmental contaminants, and nutritional deficiencies (Harrison 1986; Olsen 2003; Tully et al. 2000). In contrast to most other large clusters of beak abnormalities that have been reported in embryos or nestlings (Fox et al. 1991; Ohlendorf et al. 1986), deformities associated with avian keratin disorder develop in adult birds (Handel et al. 2010). This delayed onset suggests that deformities either are the product of a latent condition or result from exposure to etiological agent(s) over time. If avian keratin disorder is associated with nutritional problems or environmental contaminants, diet is likely to be a contributing factor. Therefore, identifying potential differences in dietary sources between affected and unaffected birds may provide valuable insights about the possible cause of beak deformities in Alaskan birds.

Black-capped chickadees consume a diverse generalist's diet that consists largely of arthropods and other invertebrates, natural seeds, and berries (Foote et al. 2010). Chickadees are common backyard birds and also feed readily on anthropogenic food sources, including sunflower seeds, peanut butter, and suet (Foote et al. 2010). These supplemental foods provide a valuable energy source during winter (Brittingham and Temple 1988; Brittingham and Temple 1992; Desrochers et al. 1988), when chickadees utilize large amounts of fat daily to support high metabolic costs (Chaplin 1974). Anthropogenic foods have also recently become of interest in the study of avian keratin disorder. Most observations of beak deformities in Alaska have been reported from urban sites where birds have access to feeders. This pattern is likely related to the limited number of human observers at non-urban sites but has also raised concern about a potential causal link between anthropogenic foods and avian keratin disorder. If feeder foods are nutritionally inadequate or somehow contaminated, it is possible that high levels of consumption could be related to the development of beak deformities.

It is also possible that beak deformities may lead to dietary shifts due to reduced functionality of the beak. Chickadees affected by avian keratin disorder display overgrowth of the upper beak, lower beak, or both, resulting in overbite, underbite or sometimes beak crossing. The severity of this condition often changes over time and deformities range from slight elongation to dramatic overgrowth and curvature (Handel et al. 2010). The effects of avian keratin disorder on individual health and fitness have not been fully assessed, but previous studies suggest that normal feeding and preening behaviors are compromised in affected birds (D'Alba et al. 2011; Handel et al. 2010).

Thus, we might expect to see dietary changes as a consequence of beak deformities, which could have significant nutritional impacts for affected birds.

In this study we investigate the potential association between diet and beak deformities in black-capped chickadees, using naturally occurring differences in carbon and nitrogen stable isotope ratios. Information that can be derived from stable isotope analysis has broad applicability to avian ecology and is particularly useful in dietary reconstruction (review in Inger and Bearhop 2008). Ratios of carbon and nitrogen isotopes differ among types of plants (C3 versus C4 photosynthetic pathways), by trophic position in a food web, and by agricultural methods; thus, they may vary between natural and anthropogenic foods and among classes of each food type (Fry 2006). The isotopic signatures of dietary items become incorporated into a consumer's tissues in a predictable manner (DeNiro and Epstein 1978; DeNiro and Epstein 1981) and provide dietary information that is integrated over time. Here we used stable isotope analysis to test for dietary differences between affected and unaffected birds and to evaluate the hypothesis that anthropogenic foods are causally related to this disorder.

4.3 Methods

4.3.1 Sample collection

Between January 2007 and March 2009, we captured wintering chickadees (November–March) at three sites in south-central Alaska (Anchorage, 61°10′N, 149°79′W; Eagle River, 61°14′N, 149°16′W; Chugiak, 61°26′N, 149°26′W) using funnel

traps and mist nets (Handel et al. 2010). All sites were dominated by mixed deciduous-coniferous woodland consisting primarily of paper birch (*Betula papyrifera*), black cottonwood (*Populus trichocarpa*), and white spruce (*Picea glauca*), and were in close proximity to human residences and known sources of supplementary foods.

We used previously established criteria of beak morphometrics in chickadees (Handel et al. 2010) to identify individuals that were affected by avian keratin disorder (nares-to-tip ≥8.5 mm or over- or underbite ≥1.0 mm). We then divided affected birds into two groups based on the severity of beak overgrowth. We assigned birds with upper or lower beak measurements <9.0 mm as "mild" cases and those ≥9.0 mm as "moderate-severe" cases. Typically, the severity of a deformity worsens over time so these categories provide a relative index of disease progression. In addition, they are correlated with functionality of the beak, with moderate-severe deformities indicative of significantly reduced function (C. Van Hemert unpublished data).

We collected blood samples from chickadees with and without beak deformities at each location. We collected 1–5 μl of whole blood into non-heparinized capillary tubes using brachial venipuncture with a sterile 27.5-ga hypodermic needle, then stored samples in 70% ethanol. Dietary integration of whole blood typically occurs over a relatively short period for small passerines (2-3 weeks; Hobson and Bairlein 2003; Pearson et al. 2003) and blood samples were therefore assumed to reflect winter diets. We banded birds upon capture and thus could ensure that each blood sample was collected from a unique individual. We aged birds as hatching year or adult based on the amount and extent of white on the rectrices (Pyle 1997). All work was completed under

the guidance of the U. S. Geological Survey and the University of Alaska Fairbanks Animal Care and Use committees (UAF Assurance #07-49).

We collected arthropods, berries, and seeds from each of the three sites during the winters of 2007–2010, selecting diet items based on our field observations and reports from other studies (Foote et al. 2010 and references therein). During winter in south-central Alaska most terrestrial arthropods occur under tree bark, where chickadees are often observed foraging (Collet 2010; C. Handel, C. Van Hemert pers. obs.). Arthropod samples primarily consisted of spiders (family Aranae) and adult insects or larvae of the orders Coleoptera and Diptera. We collected natural seeds (*B. papyrifera*) and berries (*Viburnum edule*) that remain available throughout the winter months at these sites. We also sampled human-provided sunflower seeds, peanut butter, and suet from bird feeders near the study sites and from local retailers. We identified these as the most commonly provided foods during the winter from surveys of local residents (C. Handel unpublished data). Although millet is also included in some mixed seed or suet blocks, this is not a favored food source for chickadees (Burton and Kress 1999) and thus we did not include it as a dietary endpoint in our analysis.

4.3.2 Stable isotope analysis

We analyzed samples for ratios of stable carbon ($\delta^{13}C$) and stable nitrogen ($\delta^{15}N$) at the Alaska Stable Isotope Facility at the University of Alaska Fairbanks. We dried all blood and diet samples for 72 hours in an oven at 60°C, then pulverized them with a mortar and pestle. We weighed 0.3–0.5 mg of dried, homogenized sample into a tin

capsule, which was crushed and loaded into an autosampler. All samples were combusted at 1800°C in a Costech ECS4010 elemental analyzer. We then measured the ratios of stable carbon and nitrogen by continuous flow isotope-ratio mass spectrometry using a Finnigan Delta Plus XP isotope ratio mass spectrometer with a ConFlo III combustion interface (Thermo-Finnigan Corporation). Stable isotope ratios are reported in delta (δ) notation (‰; per mil) relative to standards based on the Vienna PeeDee Belemnite for $\delta^{13}C$ measurements and atmospheric N_2 for $\delta^{15}N$ measurements. The δ values are calculated using the equation: $\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000‰$, where X is ^{13}C or ^{15}N and R is the corresponding ratio of $^{13}C/^{14}C$ or $^{15}N/^{14}N$. Repeat analyses of an internal standard (peptone) interspersed with samples demonstrated a reproducibility of ± 0.09‰ for carbon and ± 0.20‰ for nitrogen.

4.3.3 Data analysis

We tested the effects of site, deformity status and their interaction on blood $\delta^{13}C$ and $\delta^{15}N$ values using a general linear model (GLM; SAS 2008) for each isotope. Because within-group variance differed by deformity status for $\delta^{13}C$ (Levene's test; $F_{2,108} = 8.81$, $P < 0.001$), we performed this analysis on ranked data. $\delta^{15}N$ values conformed to the assumption of homogeneity of variance and thus were not transformed. We used the SIAR (stable isotope analysis in R; R Development Core Team 2007) package (Jackson et al. 2009; Parnell et al. 2008; Parnell et al. 2010) to estimate the proportional contribution of individual diet items to blood carbon and nitrogen. Resulting models identify a range of solutions for the proportion of each food item, with the median of

these solutions representing the maximum likelihood. These models require an estimate of trophic enrichment, which is the expected difference in isotope ratio between dietary items and consumer blood. We used trophic enrichment factors (± SD) of 1.7 ± 0.6‰ for $\delta^{13}C$ and 2.4 ± 0.6‰ for $\delta^{15}N$, based on previous studies of similar-sized passerines (Hobson and Bairlein 2003; Pearson et al. 2003). We used mean $\delta^{13}C$ and $\delta^{15}N$ values for the natural and anthropogenic foods (± 1 SD) and concentration-dependent models to account for the variation in C and N in different sources. Natural foods did not vary by site (all $P > 0.05$) so we grouped these for analysis. We also grouped natural seeds and berries, which had similar isotopic signatures ($\delta^{13}C$: $P = 0.71$; $\delta^{15}N$: $P = 0.12$). Due to extremely low concentration of N (<1%) in suet, only two samples were of sufficient volume to accurately estimate $\delta^{15}N$ values. To address the high variability observed in isotope values in birds with beak deformities, we used the SIARSOLO command to model dietary composition for affected individuals. This post-hoc analysis allowed us to explore possible dietary explanations for the more extreme isotopic values that were not well-represented in the group analysis.

4.4 Results

Through targeted capture efforts, we sampled 70 birds with normal beaks and 41 birds with beak deformities (27 slight; 14 moderate-severe) across the three sites. Blood samples from birds with beak deformities had lower mean $\delta^{15}N$ values than samples from birds with normal beaks (Figure 4.1; $F_{2,102} = 10.91$, $P < 0.001$). Least squares (LS) means of $\delta^{15}N$ differed significantly between the three groups (all $P < 0.05$); unaffected

birds exhibited the highest value (5.21 ± 0.10‰), followed by mildly affected birds (4.41 ± 0.16‰), and moderately-severely affected birds (3.77 ± 0.22‰). Ranked $\delta^{13}C$ values did not differ by deformity status ($F_{2,102} = 2.28$, $P = 0.11$), but samples from affected birds exhibited greater variability (Figure 4.1).

Site was a significant predictor of $\delta^{15}N$ ($F_{2,102} = 22.68$, $P < 0.001$), with a significantly higher mean for Eagle River (5.07 ± 0.15‰) compared to Anchorage (4.10 ± 0.17; $P < 0.001$‰) or Chugiak (4.22 ± 0.18‰; $P < 0.001$). Similarly, ranked $\delta^{13}C$ values differed by site ($F_{2,102} = 8.81$, $P < 0.001$), with Eagle River (-23.91 ± 0.15‰) averaging higher than Anchorage (-24.75 ± 0.17‰; $P < 0.001$) or Chugiak (-24.44 ± 0.18‰; $P = 0.03$). The interaction between site and deformity status was not significant for either isotope.

Among the food sources, suet was significantly enriched in ^{13}C. The other items exhibited similar $\delta^{13}C$ values but differed in $\delta^{15}N$ values (Figure 4.2). Arthropods were the most enriched in ^{15}N, followed by sunflower seeds and peanut butter. Natural seeds and berries had the lowest $\delta^{15}N$ values (Figure 4.2).

Results from the SIAR analysis (Figure 4.3) indicated that birds with normal beaks consumed primarily sunflower seeds and arthropods. Peanut butter, suet, and natural seeds and berries made up a smaller proportion of their diet. Compared to unaffected birds, birds with beak deformities showed a decrease in their diet in the proportion of arthropods and sunflower seeds and an increase in the proportion of natural seeds and berries and peanut butter. The group with moderate-severe beak deformities had the most pronounced decrease in the proportion of arthropods. Suet composed a

slightly lower proportion in the diet of mildly affected birds and a higher, but more variable, proportion in the diet of moderately to severely affected birds relative to birds with normal beaks.

SIARSOLO demonstrated unique dietary compositions for some affected individuals. In particular, the proportion of suet in the diet varied widely, comprising 30–40% of the diet in several affected birds with the highest $\delta^{13}C$ values, compared to almost none in others with much lower $\delta^{13}C$ values (Figure 4.4). Several other individuals with especially low $\delta^{15}N$ values demonstrated very limited consumption of arthropods and correspondingly high consumption of peanut butter and natural seeds and berries.

4.5 Discussion

Winter dietary patterns differed between black-capped chickadees with normal beaks and those afflicted with mild or moderate-severe beak deformities. These differences are consistent with the hypothesis that changes in diet are a result rather than a cause of avian keratin disorder. Although some individuals with beak deformities relied heavily on feeder foods, we did not find evidence of an anthropogenic diet source shared by all affected birds. Changes in diet due to reduced function of the beak may have important nutritional implications for affected birds, particularly for those with pronounced deformities.

The relative contribution of arthropods to the diet decreased with increasing severity of beak deformities, and this pattern was likely due to reduced beak function. During winter, chickadees normally obtain a significant source of animal protein by

foraging under the bark of trees for overwintering adult spiders and insects, and their larvae or eggs (Foote et al. 2010). They also rely heavily on food caches, including arthropods collected earlier in the year (Brodin and Grubb 2005). Such resources are likely to be inaccessible to birds with severely deformed beaks, resulting in reduction of critical sources of dietary protein.

The reduction of sunflower seeds and increase in peanut butter and natural seeds and berries in the diets of affected birds may be similarly related to the degree to which these foods have to be manipulated prior to consumption. Unhulled black oil sunflower seeds are commonly provided at bird feeders, but birds must remove the outer shell to access the seed inside, a task that poses a significant challenge if the beak is overgrown. Peanut butter requires no manipulation and is often provided at residences where affected birds have been observed (C. Handel unpublished data). Some natural seeds and berries remain on branches or, in the case of birch seeds, are found on the snow surface and thus are relatively accessible throughout the winter. We frequently observed altered feeding behaviors in affected birds, including turning the head parallel to the ground and eating out of the side of the beak (Figure 4.5). Chickadees with beak deformities also often foraged extensively on the ground and beneath bird feeders for seed pieces that were dropped (Figure 4.5; Handel et al. 2010). Such behavior is not common among birds with normal beaks (Foote et al. 2010) and likely places the birds at higher risk of predation.

Commercially-available suet was of particular *a priori* interest due to potentially high levels of lipophilic contaminants and growth hormones (Fries 1995; Hallikainen and

Vartiainen 1997; Waltner-Toews and McEwen 1994). Several individuals with abnormal beaks appeared to specialize on suet, but others consumed little to none. The lack of consistent suet use among deformed birds suggests that it is not likely to be associated with the development of beak deformities in chickadees.

Birds with moderate-severe deformities exhibited the most significant dietary differences, suggesting that such changes are a consequence rather than a cause of avian keratin disorder. Our documentation of several affected birds at remote sites far from human habitations also supports this assertion. We previously reported a single chickadee with a grossly elongated beak on the Kenai National Wildlife Refuge >15 km from any known human residences or anthropogenic food sources (Handel et al. 2010). We later detected two other birds with beak deformities in this area, each at a relative large distance (>15 km) from the first capture site and any known human habitations (C. Van Hemert unpublished data). Individual chickadees have restricted home-ranges and high site fidelity (Glase 1973; Odum 1942; Smith 1991); thus, the presence of affected birds at remote sites suggests that deformities may in fact develop in the absence of bird feeders and at relatively large distances from human communities.

Similar dietary patterns relative to deformity status across the three sampling locations suggest a consistent response of chickadees to beak dysfunction, despite some spatial variation in isotopic values of chickadee blood samples. The higher values of $\delta^{13}C$ and $\delta^{15}N$ from chickadees sampled at Eagle River may reflect site-specific isotopic variability. In particular, salmon (enriched in ^{15}N) spawn in this river and could influence isotopic values of the adjacent terrestrial system (Bilby et al. 1996; Helfield and Naiman

2001; Reimchen et al. 2003). Alternatively, differences in availability of natural or anthropogenic foods might explain variability across sites. For example, suet, which is enriched in ^{13}C, is regularly provided in feeders at the Eagle River site (C. Van Hemert pers. obs.) and may be less consistently available at other sites. Although we did not detect a difference in natural dietary sources between sites, relatively small sample sizes may have limited the sensitivity of this assessment.

The use of stable isotopes is a valuable tool for dietary estimation, but has certain limitations and requires several assumptions. The precision with which diet can be characterized using isotopic methods is constrained by the isotopic differences among and variability within specific food items. In this study, isotopic overlap between some sources, for example, sunflower seeds and arthropods, and peanut butter and natural seeds and berries, resulted in fairly broad credibility intervals around estimates generated by SIAR analysis. The use of experimental anthropogenic (supplementary) foods that are heavily isotopically labeled can circumvent this problem (Robb et al. 2011), but is not feasible for areas where there is also high availability of unlabeled supplementary foods such as our urban sites. Despite these sources of uncertainty, we were able to quantify dietary shifts associated with beak deformity that were nutritionally significant and consistent with field observations of beak function. Dietary items also varied widely in lipid content, which could affect the extent to which they contributed carbon to blood, a protein-based tissue. For two reasons we considered our use of a concentration-dependent mixing model a better choice to account for these variations than lipid-extraction of diet items (e.g., Post et al. 2007). First, chickadees specialize during the

winter on lipid-rich foods, such as suet, and lipid-extracting such foods would have essentially removed them from analysis. Second, carbon from dietary lipid has been shown to make major contributions to tissue proteins in passerines, particularly when fed high-fat and low-protein diets similar to those of wintering chickadees (Herrera et al. 2009; Podlesak and McWilliams 2006; Podlesak and McWilliams 2007).

Results from this study provide valuable insights about the relationship between beak deformities and diet in black-capped chickadees. Changes in dietary composition have important implications for avian health and nutrition, especially given the high prevalence of this disorder in multiple species and across a growing geographic area (Handel et al. 2010; Van Hemert and Handel 2010). Nutritional and behavioral consequences of dietary shifts could contribute to high mortality rates and compromised fitness associated with beak deformities in chickadees (Handel et al. 2006; Handel et al. 2010). Our results also suggest that we should reduce emphasis on anthropogenic food sources as likely causative factors and that other etiologies may be more likely. Epidemiological and demographic analyses will be important components in future efforts to identify the cause and consequences of this widespread problem in wild birds.

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necessarily represent the official views of NCRR or NIH. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

4.7 Literature Cited

Bilby RE, Fransen BR, Bisson PA (1996) Incorporation of nitrogen and carbon from spawning coho salmon into the trophic system of small streams: evidence from stable isotopes. Can J Fish Aquat Sci 53:164-173

Brittingham MC, Temple SA (1988) Impacts of supplemental feeding on survival rates of black-capped chickadees. Ecology 69:581-589

Brittingham MC, Temple SA (1992) Use of winter bird feeders by black-capped chickadees. J Wildl Manage 56:103-110

Brodin A, Grubb TC, Jr. (2005) Hippocampal volume does not correlate with food-hoarding rates in the black-capped chickadee (*Poecile atricapillus*) and willow tit (*Parus montanus*). Auk 122:819-828

Burton R, Kress SW (1999) The Audubon Backyard Birdwatcher: Birdfeeders and Bird Gardens. Thunder Bay Press, San Diego, California

Chaplin SB (1974) Daily energetics of the black-capped chickadee, *Parus atricapillus*, in winter. J Comp Physiol B 89:321-330

Collet DM (2010) Insects of South-central Alaska. Kenai Watershed Forum, Kenai, Alaska

D'Alba L, Van Hemert C, Handel CM, Shawkey MD (2011) A natural experiment on the condition-dependence of achromatic plumage reflectance in black-capped chickadees. PLoS ONE 6:e25877

DeNiro MJ, Epstein S (1978) Influence of diet on the distribution of carbon isotopes in animals. Geochim Cosmochim Acta 42:495-506

DeNiro MJ, Epstein S (1981) Influence of diet on the distribution of nitrogen isotopes in animals. Geochim Cosmochim Acta 45:341-351

Desrochers A, Hannon SJ, Nordin KE (1988) Winter survival and territory acquisition in a northern population of black-capped chickadees. Auk 105:727-736

Foote JR, Mennill DJ, Ratcliffe LM, Smith SM (2010) Black-capped Chickadee (*Poecile atricapillus*). In: Poole A (ed) Birds N Am. Cornell Lab of Ornithology, Ithaca, NY

Fox GA, Collins B, Hayakawa E, Weseloh DV, Ludwig JP, Kubiak TJ, Erdman TC (1991) Reproductive outcomes in colonial fish-eating birds: a biomarker for developmental toxicants in Great Lakes food chains. II. Spatial variation in the occurrence and prevalence of bill defects in young double-crested cormorants in the Great Lakes, 1979-1987. J Great Lakes Res 17:158-167

Fries GF (1995) A review of the significance of animal food products as potential pathways of human exposures to dioxins. J Anim Sci 73:1639-1650

Fry B (2006) Stable Isotope Ecology. Springer, New York, NY

Glase JC (1973) Ecology of social organization in the black-capped chickadee. Living Bird 12:235-267

Hallikainen A, Vartiainen T (1997) Food control surveys of polychlorinated dibenzo-p-dioxins and dibenzofurans and intake estimates. Food Additives and Contaminants 14:355-366

Handel CM, Pajot LM, Matsuoka SM, Trust KA, Stotts JM, Terenzi J, Talbot SL (2006) Potential role of environmental contaminants in the pathology of beak deformities among black-capped chickadees in south-central Alaska. Unpublished Final Report. USGS Alaska Science Center, Anchorage, Alaska

Handel CM, Pajot LM, Matsuoka SM, Van Hemert C, Terenzi J, Talbot SL, Mulcahy DM, Meteyer CU, Trust KA (2010) Epizootic of beak deformities among wild birds in Alaska: an emerging disease in North America? Auk 127:882-898

Harrison GJ (1986) Disorders of the integument. In: Harrison GJ, Harrison LR (eds) Clinical Avian Medicine and Surgery. W. B. Saunders Co., Philadelphia, Pennsylvania., pp 397-407

Helfield JM, Naiman RJ (2001) Effects of salmon-derived nitrogen on riparian forest growth and implications for stream productivity. Ecology 82:2403-2409

Herrera M, L. Gerardo, M. Rodríguez GM, Hernández P (2009) Sources of assimilated protein in a specialized tropical frugivorous bird, the yellow-throated euphonia (*Euphonia hirundinacea*). Auk 126:175-180

Hobson K, Bairlein F (2003) Isotopic fractionation and turnover in captive garden warblers (*Sylvia borin*): implications for delineating dietary and migratory associations in wild passerines. Can J Zool 81:1630-1635

Inger R, Bearhop S (2008) Review: applications of stable isotope analyses to avian ecology. Ibis 150:447-461

Jackson AL, Inger R, Bearhop S, Parnell A (2009) Erroneous behaviour of MixSIR, a recently published Bayesian isotope mixing model: a discussion of Moore & Semmens (2008). Ecol Lett 12:E1-E5

Odum EP (1942) Annual cycle of the black-capped chickadee-3. Auk 59:499-531

Ohlendorf HM, Hoffman DJ, Saiki MK, Aldrich TW (1986) Embryonic mortality and abnormalities of aquatic birds: apparent impacts of selenium from irrigation drainwater. Sci Total Environ 52:49-63

Olsen GH (2003) Oral biology and beak disorders of birds. Vet Clin N Am Exot Anim Pract 6:505-521

Parnell A, Inger R, Bearhop S, Jackson AL (2008) SIAR: Stable Isotope Analysis in R. Available at: http://cran.r-project.org/web/packages/siar/index.html

Parnell AC, Inger R, Bearhop S, Jackson AL (2010) Source partitioning using stable isotopes: coping with too much variation. PLoS ONE 5:e9672

Pearson SF, Levey DJ, Greenberg CH, Martinez Del Rio C (2003) Effects of elemental composition on the incorporation of dietary nitrogen and carbon isotopic signatures in an omnivorous songbird. Oecologia 135:516-523

Podlesak DW, McWilliams SR (2006) Metabolic routing of dietary nutrients in birds: Effects of diet quality and macronutrient composition revealed using stable isotopes. Phys Biochem Zool 79:534-549

Podlesak DW, McWilliams SR (2007) Metabolic routing of dietary nutrients in birds: effects of dietary lipid concentration of $\delta^{13}C$ and its ecological implications. Auk 124:916-925

Post DM, Layman CA, Arrington DA, Takimoto G, Quattrochi J, Montana CG (2007) Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analyses. Oecologia 152:179-189

Pyle P (1997) Identification Guide to North American Birds: a Compendium of Information on Identifying, Ageing, and Sexing 'Near-Passerines' and Passerines in the Hand. Slate Creek Press, Bolinas, CA

R Development Core Team (2007) R: A language and environment for statistical computing. R Foundation for Statistical Computing,Vienna, Austria. Available at: http://www.R-project.org

Reimchen TE, Mathewson D, Hocking MD, Moran J, Harris D (2003) Isotopic evidence for enrichment of salmon-derived nutrients in vegetation, soil, and insects in riparian zones in coastal British Columbia. Am Fish Soc Symp 34:59-69

Robb GN, McDonald RA, Inger R, Reynolds SJ, Newton J, McGill RAR, Chamberlain DE, Harrison TJE, Bearhop S (2011) Using stable-isotope analysis as a technique for determining consumption of supplementary foods by individual birds. Condor 113:475-482

SAS Institute (2008) Version 9.2. Cary, North Carolina

Smith SM (1991) The Black-capped Chickadee: Behavioral Ecology and Natural History. Cornell University Press, Ithaca, New York

Tully TN, Jr., Lawton MPC, Dorrestein GM (eds) (2000) Handbook of Avian Medicine, 2nd edn. Butterworth-Heinemann, Elsevier Science Ltd., New York

Van Hemert C, Handel CM (2010) Beak deformities in northwestern crows: evidence of a multispecies epizootic. Auk 127:746-751

Waltner-Toews D, McEwen SA (1994) Residues of hormonal substances in foods of animal origin: a risk assessment. Prev Vet Med 20:235-247

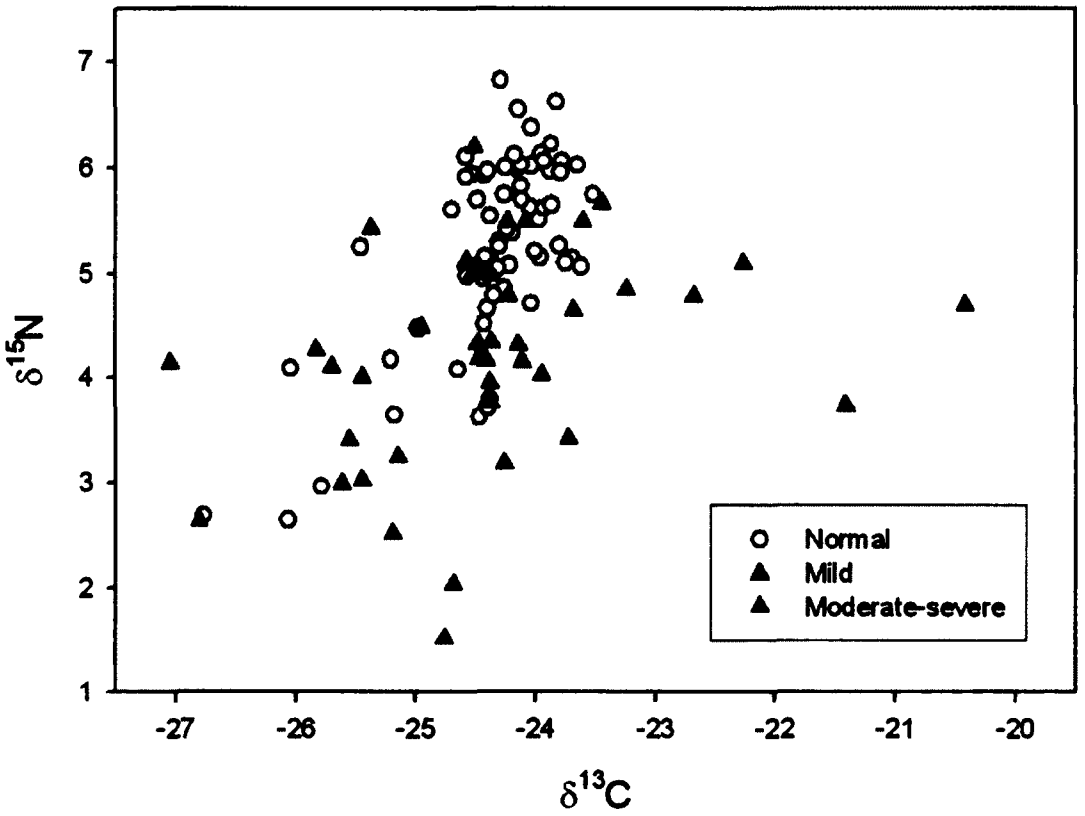

Figure 4.1 Stable isotope ratios of carbon and nitrogen in whole blood of black-capped chickadees with normal beaks (white circles), mild beak deformities (grey triangles), and moderate-severe beak deformities (black triangles) from south-central Alaska between January 2007 and March 2009.

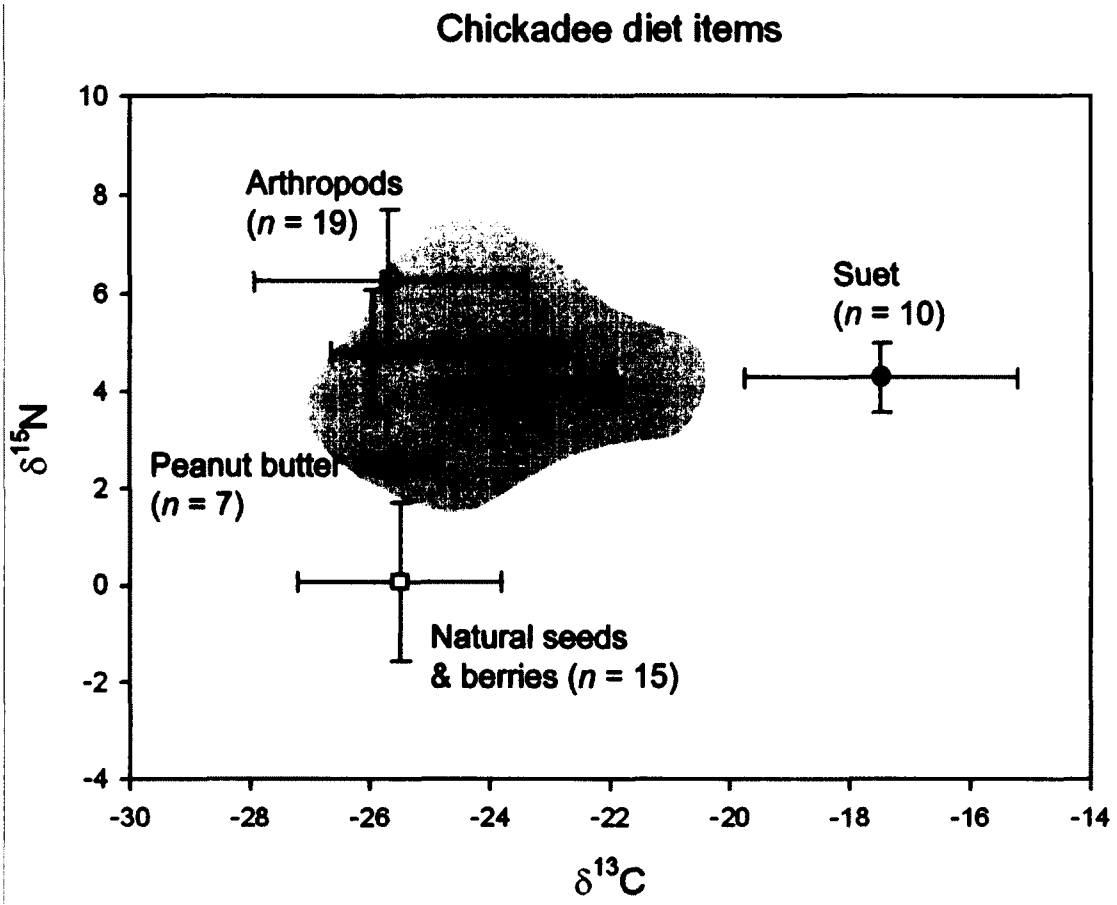

Figure 4.2 Stable isotope ratios (mean ± 1 SD) of carbon and nitrogen for potential food sources of black-capped chickadees, collected in south-central Alaska during winters 2007-2010. Values adjusted with trophic enrichment factor of 1.7‰ for $\delta^{13}C$ and 2.4‰ for $\delta^{15}N$. Grey cloud shows approximate range of values for chickadee blood samples.

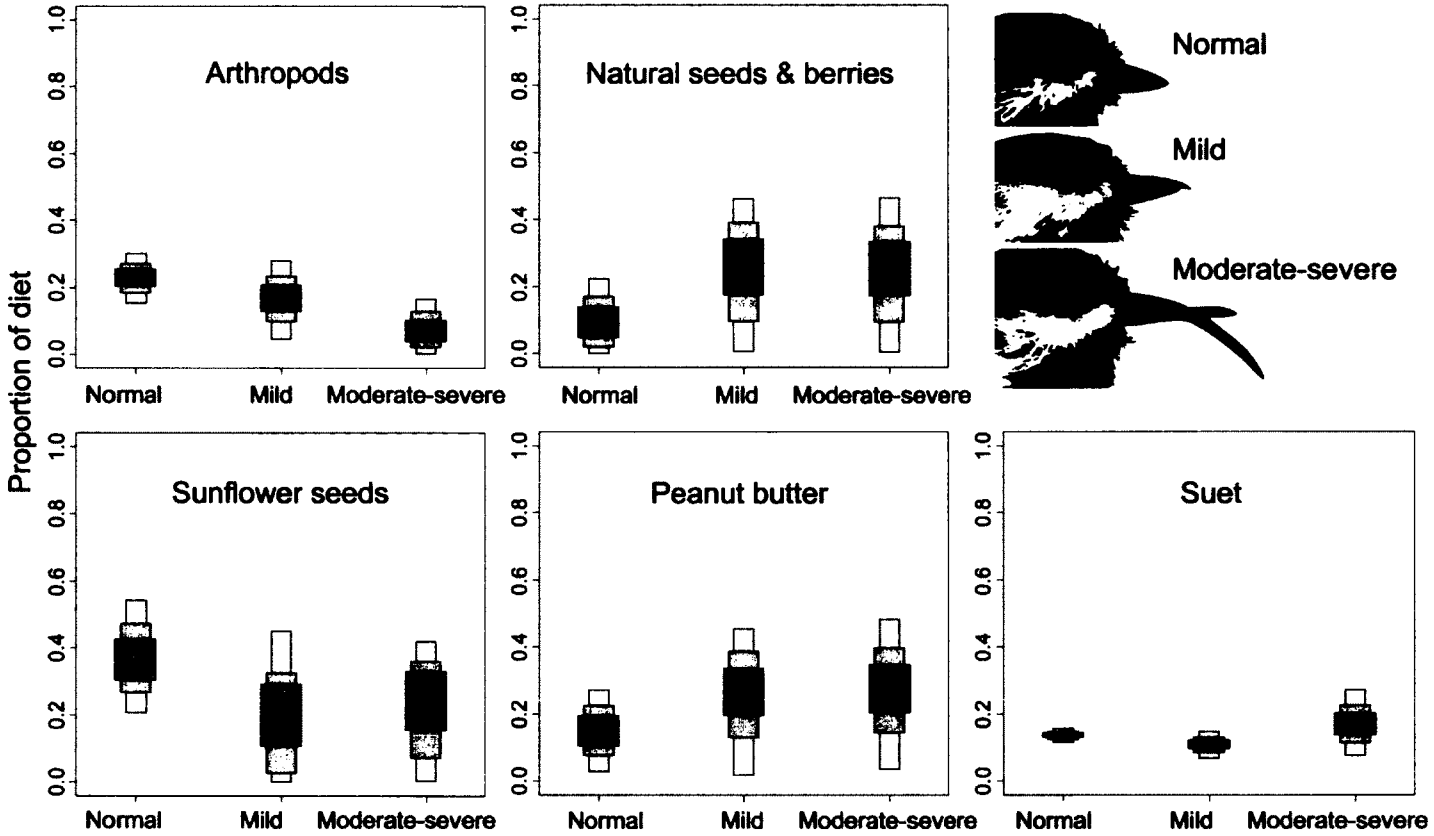

Figure 4.3 Relative proportion of winter food sources of black-capped chickadees in Alaska. Plots show 50%, 75%, and 95% credibility intervals of the maximum likelihood values estimated using SIAR for chickadees with normal beaks (n =70), mild beak deformities (n = 27), and moderate-severe beak deformities (n = 14).

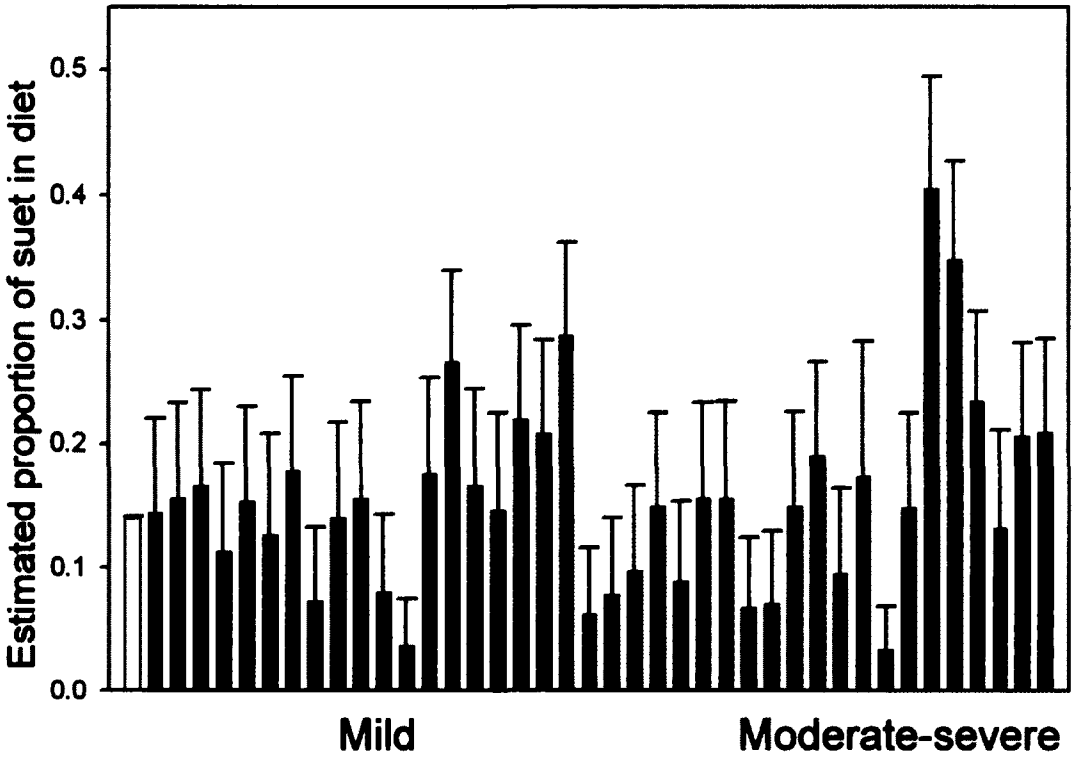

Figure 4.4 Estimated proportion of suet in the winter diet of individual black-capped chickadees affected by avian keratin disorder. Bars show mean ± 1 SD of the maximum likelihood value estimated using SIAR for birds with mild beak deformities (gray bars) and moderate-severe beak deformities (black bars). Reference value of mean ± 1 SD for all birds with normal beaks shown on left (white bar).

Figure 4.5 Examples of black-capped chickadees with beak deformities. Affected birds often rely on anthropogenic food sources and may exhibit altered feeding behavior to accommodate beak overgrowth. A) Chickadee foraging on sunflower seed pieces in snow beneath bird feeder (photograph by Ken Whitten). B) Chickadee turning head sideways to feed on peanut butter (photograph by Richard Flanders).

CHAPTER 5. BEAK DEFORMITIES IN NORTHWESTERN CROWS: EVIDENCE OF A MULTI-SPECIES EPIZOOTIC[1]

5.1 ABSTRACT

Beak abnormalities are rare among adult birds and, typically, are not widespread in a given population, within a region, or across multiple species. A high concentration of beak deformities was recently documented among Black-capped Chickadees (*Poecile atricapillus*) and other resident avian species in Alaska. Here, we describe a parallel condition in Northwestern Crows (*Corvus caurinus*), signaling the emergence of a multi-species epizootic. Based on trapping of Northwestern Crows (n = 186) at six sites in Alaska during 2007 and 2008, we estimated prevalence of beak deformities in adults to be 16.9 ± 5.3%, the highest rate of gross deformities ever recorded in a wild bird population. Prevalence varied among sites and was as high as 36% on the Kenai Peninsula, suggesting possible epizootic clusters. We also documented beak abnormalities in an additional 148 Northwestern Crows in south-central and southeastern Alaska and in 64 crows near Vancouver, British Columbia, and Puget Sound, Washington, a region where both Northwestern and American (*C. brachyrhynchos*) crows occur. The increase in frequency and distribution of crows observed with abnormal beaks throughout the Pacific Northwest since the late 1990s indicates a geographic expansion of this problem. Affected crows exhibited elongated and often crossed beaks that were morphologically similar to deformities documented in Black-

[1] Van Hemert, C., and C. M. Handel. 2010. Beak deformities in Northwestern Crows: Evidence of a multi-species epizootic. *Auk* 127: 746-51.

capped Chickadees and other species in Alaska over approximately the same time period. Additional research is needed to determine the etiology and potential adverse effects on bird populations affected by this disorder.

5.2 INTRODUCTION

High rates of gross deformities in wildlife populations often serve as indicators of underlying environmental health problems[1,2,3,4]. An epizootic of beak deformities, referred to as avian keratin disorder, has been documented among Black-capped Chickadees (*Poecile atricapillus*) in Alaska during the past decade and afflicts an average of 6.5% of the adult population annually[5]. In affected chickadees, the keratin layer of the beak becomes overgrown, resulting in noticeably elongated, often crossed presentation, sometimes accompanied by abnormal skin and feathers. Morphologically similar deformities have recently appeared in other, primarily resident Alaskan species, including Northwestern Crows (*Corvus caurinus*) throughout their range in coastal Alaska[5]. Beak abnormalities have also been documented in coastal British Columbia and Washington, where the ranges of Northwestern and American crows (*C. brachyrhynchos*) overlap[6,7]. The presence of deformities in crows suggests that this epizootic is not restricted to a discrete geographic area or a single species and that etiological agents may occur across a broad environmental gradient, affecting both terrestrial and coastal systems. The primary objectives of our study were to estimate prevalence and describe the gross morphology of beak deformities in Alaskan Northwestern Crow populations relative to recent findings for Black-capped Chickadees. We also investigated geographic distribution of the

problem by compiling and analyzing confirmed reports of beak abnormalities in all species of crows in North America.

5.3 PREVALENCE

Between March 2007 and April 2008, we captured, measured, and examined a total of 186 Northwestern Crows at six sites in coastal Alaska to characterize and estimate prevalence of beak deformities in the population. We documented 19 adult Northwestern Crows with beaks classified as deformed based on our prediction interval criteria (see Supplementary Methods and Table 5.S1 for further information). We estimated an overall prevalence of 16.9 ± 5.3% among adults across all sites (Table 5.1), which exceeds an expected background level of deformity by more than 30 times[8,9].

Together, the prevalence of beak deformities among adult Northwestern Crows and that among Black-capped Chickadees (6.5%[5]) in Alaska represent the highest rates of gross deformities ever recorded in wild bird populations. It is possible that these estimates of prevalence are positively biased because trapping for both species relied on baiting with supplemental food, which might attract a greater proportion of birds whose abnormal beaks impair natural foraging. However, both crows[6,10,11] and chickadees[12,13] in their normal state are generalist predators that are highly flexible behaviorally. These natural traits and the large numbers of birds with grossly deformed beaks observed throughout the region both suggest that, even if a trap-related bias occurred, it was unlikely to account for the unusually high prevalence documented among crows and chickadees.

We found that the occurrence of beak deformities in adult crows was independent of sex ($P = 0.806$, $z = -0.245$, $n = 135$) but differed between age classes as we did not observe any evidence of beak deformities in juveniles captured ($n = 50$). Age- and sex-related patterns of this epizootic appear to be very similar between chickadees and crows. Among chickadees, deformities occur almost exclusively in adults and at only a slightly higher rate among females than males[5].

We detected geographic variation in prevalence of deformities in crows across our study locations ($P = 0.043$, $z = 2.024$, $n = 135$; Table 5.1), with extremely high rates in Kenai (36%) and Seward (33%). Although sample sizes of individuals trapped per site were relatively small, differences in prevalence between sites suggest the occurrence of epizootic clusters, as has been noted for chickadees[5]. Morphologically similar beak deformities also occur in a wide suite of other resident species and we have documented Black-capped Chickadees and as many as eight other species with beak deformities at or near each of our Northwestern Crow study locations (C. M. Handel unpubl. data). Seward and Kenai may represent relative "hotspots" of this disorder for Northwestern Crows, although possible reasons for such spatial distribution are currently unknown. These two sites are the closest of our sampling locations in geographic proximity to the known epicenter of avian keratin disorder among Black-capped Chickadees[5] and abnormalities in other taxa have also been observed in this region of Alaska. Elevated prevalence of eye and skeletal abnormalities of unknown etiology among Wood Frogs (*Rana sylvatica*) was documented on the Kenai National Wildlife Refuge, near the city of Kenai[14]. Although no link has been established between deformities in other taxa and

avian keratin disorder, spatial overlap of these abnormalities may warrant concern about underlying environmental factors in this region.

5.4 MORPHOLOGY OF DEFORMITIES

We observed three distinct forms of deformities: elongation of the upper beak, elongation of the lower beak, and elongation of both the upper and lower beaks, all of which involve some form of overgrowth and apparently reflect different presentations of avian keratin disorder in crows (Fig. 5.1). Based on measurements from our study and other reports compiled in our observation database of beak deformities in Northwestern and American crows, the most common morphology associated with this condition involves overgrowth of the upper beak (Fig. 5.1C,D, Table 5.2). Only four of the individuals that we captured in this study had an abnormally elongated lower beak and a pronounced underbite relative to the upper beak (Fig. 5.1E,F). Among birds with normal beaks, minor (<5 mm) overbite was relatively common but we did not observe any measurable underbite. Four captured crows exhibited elongation of both the upper and lower beaks (Table 5.2). Other abnormal physical characteristics of the beak often accompanied gross deformities in affected individuals. Among crows captured with deformed beaks, 42% (n = 8) had a gap of >0.5 mm between the upper and lower beaks and 21% (n = 4) showed a lateral offset, which resulted in crossed tips (cf. Fig. 5.1A,B). Crows with normal beaks exhibited gaps >0.5 mm (16%; n =18) and crossing (2%; n = 2) less frequently. Irregularities of the keratin covering the beak (rhamphotheca), including serrated edges, ridges, thickening, and flattening of the tomium, occurred in 84% (n = 16)

of deformed beaks in contrast to only 7% (n = 8) of normal beaks. Beak deformities in Black-capped Chickadees and other affected species in Alaska demonstrate analogous morphologies, highlighting similarities in expression of avian keratin disorder across species[5].

We documented several other conditions that affect keratinized tissues in crows with abnormal beaks, including abnormally long and curled claws (11%; n = 2); dermatoses, observed as dry, flaky, and/or reddened skin on the head and abdomen (16%; n = 3); leg scales that appeared thickened and sloughed easily (11%; n = 2); and evidence of ectoparasites (21%; n = 4). In birds with normal beaks, we noted abnormal claws (2%; n = 2), dermatoses (3%; n = 3), and ectoparasites (3%; n = 4) rarely and observed no evidence of abnormal leg scales. Two individuals with beak deformities captured in this study also had slightly abnormal plumage coloration, including white or reddish-brown feathers in the loral and occipital regions. A subset of chickadees with beak deformities exhibited similar abnormalities of keratinized tissues[5]. Presence of elongated claws, dermatoses, and abnormal feathers and leg scales among affected crows and chickadees suggests the possibility of a systemic keratin disorder in which an overgrown beak is the most grossly evident sign. Alternatively, some of these conditions could result from altered function of abnormal beaks, and may be related to compromised preening or feeding behavior, or reduced nutritional status.

5.5 NUMBERS AND DISTRIBUTION OF DEFORMITIES

Based on review of all records of deformities from North America[5], we documented observations of 148 individual Northwestern Crows with grossly elongated or crossed beaks in Alaska between the 1980s (specific dates unknown) and 2009, with most observed since 1997 (Figs. 5.2 and 5.3). We also confirmed reports of 64 crows from Washington and British Columbia (2001–2009) but were unable to determine whether they were Northwestern or American Crows due to overlapping ranges. In comparison, we received only five reports of abnormal beaks among crows outside of this region: one American Crow from Ontario in 2001, one American Crow from Quebec in 2005, and three American or Fish (*C. ossifragus*) crows from Florida between 2005 and 2007 (due to overlapping ranges, we could not conclusively determine species). These records, in combination with findings for Black-capped Chickadees[5], suggest that avian keratin disorder occurs across multiple species and habitat types, but is currently concentrated in Alaska and other areas of the Pacific Northwest. Additional sampling of crow populations outside of the Pacific Northwest and examination of recently collected museum specimens from across North America would help to verify this apparent geographic pattern. Interannual patterns of documented deformities as well as comparison between our data and historical museum specimens (C. Van Hemert unpubl. data) and live capture records[15] (R. Ha unpubl. data) indicate that avian keratin disorder is a relatively new phenomenon among Northwestern Crows. Greater observer effort may have contributed in part to an increase in sightings over the past decade, but a dearth of published records or anecdotal reports of gross beak deformities prior to the late 1990s

suggests recent emergence of this problem. Among chickadees, a marked increase in prevalence of abnormal beaks has been observed over approximately the same time period[5], showing nearly synchronous timing of this disorder in the two species.

Despite similarities in epizootic patterns among crows and chickadees, there also appears to be some discrepancy in the geographic distribution between the two species, with occurrence south of Alaska being relatively more common among crows than chickadees. Based on our compilation of observations of beak deformities from across the continent, affected crows occur with much greater frequency in British Columbia and Washington than affected chickadees. Only 31 Black-capped Chickadees with beak abnormalities were documented anywhere outside of Alaska, including 5 from the Puget Sound area of Washington, compared with more than 2,000 affected individuals within Alaska[5]. Both chickadees and crows are gregarious and common visitors to feeders and other human food sources and it is unlikely that large numbers of grossly abnormal beaks would go unnoticed in either species within populated areas.

Reports of beak deformities in crows occurred earlier in Alaska than in more southern parts of their coastal range, suggesting an increase in the regional distribution of avian keratin disorder. Affected Black-capped Chickadees have also been reported across a growing geographic area within Alaska[5]. If movements of individual birds contribute to the transmission of this condition, differences between species in geographical spread of deformities might reflect differences in their scales of movement. Individual chickadees have restricted ranges, averaging 10–15 ha[16,17,18] and distances between breeding and wintering sites are typically less than 1 km[19]. In comparison,

although movement patterns among Northwestern Crows, particularly in Alaska, have not been well described, re-sightings of color-marked individuals from this study indicate that movements of >40 km are not unusual. In British Columbia, median distances for both juvenile dispersal and seasonal movements were approximately 13 km, but ranged as far as 177 km[6]. A better understanding of relatedness between Alaskan and more southern populations of crows would help determine the degree to which movement patterns could contribute to exposure to or transfer of etiological agents.

Although the etiology of the deformities is not yet known, investigating occurrence across multiple species provides insights about possible causes. The similarity between crows and chickadees in physical characteristics of the deformities and geographic distribution supports the hypothesis of a common etiology. Chickadees, crows, and other resident species seem to be disproportionately affected along the Pacific Northwest coast relative to the rest of North America, although many individuals with beak deformities have also been observed in interior Alaska[5]. This distributional pattern suggests that factors unique to the region may contribute to occurrence of avian keratin disorder. However, if causative agents are environmental in origin, such as anthropogenic toxicants or regional nutrient limitations, they would appear to be relatively diffuse and not easily traceable to specific locations or sources. Affected areas are geographically widespread, many of the affected populations are relatively isolated, and no unifying features (such as habitat type, extent of human development, proximity to coast, etc.) have been identified. Sampling of crows in areas isolated from human communities would help confirm whether birds with beak deformities occur away from

anthropogenic influences, including food resources, potential point-source environmental contaminants, or other localized factors, as has been suggested for Black-capped Chickadees[5]. If infectious disease or parasites are implicated in the deformities, we expect the agent(s) to be transient across a broad geographic area. Because both chickadees and crows are year-round residents with generally restricted seasonal movements between wintering and breeding areas, it is unlikely that large-scale or migratory movements of individuals of either species could have been directly responsible for such transport.

Lack of knowledge about causative factors and subsequent physiological changes associated with beak deformities emphasizes the need for additional research into the pathology of avian keratin disorder. Without a diagnostic test, we are limited in our ability to detect deformities in wild populations and must rely on observation of gross signs. As such, we cannot identify individuals early in the disease phase or birds with incipient deformities. Similarly, a complete morphological assessment, including histological examination of affected tissues, is necessary to determine whether the same physiological and cellular changes are responsible for deformities in both chickadees and crows. Birds with severe beak overgrowth clearly experience functional limitations, including difficulty feeding and preening, and may also be subject to other underlying problems associated with abnormal keratin growth. Additional research, including investigation of pathology and possible mechanisms associated with gross beak deformities, is required in order to develop a comprehensive understanding of this multi-species epizootic and its potential implications for avian populations.

5.6 METHODS SUMMARY

As part of a larger study of beak deformities in Alaska, we began in 1998 to solicit published and unpublished information to document the occurrence and distribution of North American crow species with apparently abnormal beaks, excluding those resulting from trauma or easily identifiable diseases such as avian pox. We searched the published literature, presented requests for information to national and Canadian media, collaborated with other researchers conducting studies on crows in North America, and monitored on-line avian resources[5] . We pooled observations of Northwestern and American crows from British Columbia and Washington, where their ranges overlap and they may hybridize[6,7]. We estimated the prevalence of beak deformities in Northwestern Crow populations during late winter in 2007 and 2008 at six accessible coastal sites across south-central and southeastern Alaska: Seward (60.11°N, 149.44°W), Kenai (60.55°N, 151.23°W), Homer (59.64°N, 151.54°W), Valdez (61.12°N, 146.35°W), Haines (59.23°N, 135.44°W), and Juneau (58.38°N, 134.64°W; Fig. 5.2). These sampling sites support small to moderate-sized human population centers ranging from fewer than 3,000 to more than 30,000 residents, host relatively large numbers of crows ($n > 50$), and have both natural intertidal prey (mussels and other marine invertebrates) and anthropogenic food items (discarded fish waste and urban refuse) seasonally available to crows. Given the lack of historical data on beak morphometrics, we developed an iterative statistical method using measurements from the relatively large sample of apparently normal birds in our study ($n = 115$) to identify prediction intervals

beyond which a beak would be considered deformed. Please see Supplementary Methods for additional details about field and analytical methods.

5.7 LITERATURE CITED

1. Ohlendorf, H. M., D. J. Hoffman, M. K. Saiki, and T. W. Aldrich. 1986. Embryonic mortality and abnormalities of aquatic birds: Apparent impacts by selenium from irrigation drainwater. Science of the Total Environment 52:49–63.

2. Ludwig, J. P., H. Kurita Matsuba, H. J. Auman, M. E. Ludwig, C. L. Summer, J. P. Glesy, D. E. Tillitt, and P. D. Jones. 1996. Deformities, PCBs, and TCDD-equivalents in Double-crested Cormorants (*Phalacrocorax auritus*) and Caspian Terns (*Hydroprogne caspia*) of the upper Great Lakes 1986–1991: Testing a cause-effect hypothesis. Journal of Great Lakes Research 22:172–197.

3. Johnson, P. T. J., J. M. Chase, K. L. Dosch, R. B. Hartson, J. A. Gross, D. J. Larson, D. R. Sutherland, and S. R. Carpenter. 2007. Aquatic eutrophication promotes pathogenic infection in amphibians. Proceedings of the National Academy of Sciences 104:15781–15786.

4. Sun, P. L., W. E. Hawkins, R. M. Overstreet, and N. J. Brown-Peterson. 2009. Morphological deformities as biomarkers in fish from contaminated rivers in Taiwan. International Journal of Environmental Research and Public Health 6:2307–2331.

5. Handel , C. M., L. M. Pajot, S. M. Matsuoka, C. Van Hemert, J. Terenzi, S. L. Talbot, D. M. Mulcahy, C. U. Meteyer, and K. A. Trust. 2010. Epizootic of beak

deformities among wild birds in Alaska: An emerging disease in North America? Auk 127: 882–898.

6. Verbeek, N. A. M., and R. W. Butler. 1999. Northwestern Crow (*Corvus caurinus*). *In* The Birds of North America, no. 407 (A. Poole and F. Gill, Eds.). The Birds of North America, Inc., Philadelphia, Pennsylvania.

7. Verbeek, N. A. M., and C. Caffrey. 2002. American Crow (*Corvus brachyrhynchos*). *In* The Birds of North America, no. 647 (A. Poole and F. Gill, Eds.). The Birds of North America, Inc., Philadelphia, Pennsylvania.

8. Hicks, L. E. 1934. Individual and sexual variations in the European Starling. Bird-Banding 5:103–118.

9. Craves, J. A. 1994. Passerines with deformed bills. North American Bird Bander 19:14–18.

10. Peterson, C. A., S. L. Lee, and J. E. Elliott. 2001. Scavenging of waterfowl carcasses by birds in agricultural fields of British Columbia. Journal of Field Ornithology 72:150–159.

11. O'Brien, E. L., A. E. Burger, and R. D. Dawson. 2005. Foraging decision rules and prey species preferences of Northwestern Crows (*Corvus caurinus*). Ethology 111:77–87.

12. Smith, S. M. 1991. The Black-capped Chickadee: Behavioral Ecology and Natural History. Cornell University Press, Ithaca, New York.

13. Wilson, W. H., Jr. 2001. The effects of supplemental feeding on wintering Black-capped Chickadees (*Poecile atricapilla*) in central Maine: Population size and individual responses. Wilson Bulletin 113:65–72.

14. Reeves, M. K., C. L. Dolph, H. Zimmer, R. S. Tjeerdema, and K. A. Trust. 2008. Road proximity increases risk of skeletal abnormalities in Wood Frogs from National Wildlife Refuges in Alaska. Environmental Health Perspectives 116:1009–1014.

15. Johnston, D. W. 1961. The Biosystematics of American Crows. University of Washington Press, Seattle.

16. Odum, E. P. 1942. Annual cycle of the Black-capped Chickadee—3. Auk 59:499–531.

17. Glase, J. C. 1973. Ecology of social organization in the Black-capped Chickadee. Living Bird 12:235–267.

18. Smith, S. M. 1993. Black-capped Chickadee (*Poecile atricapillus*). *In* The Birds of North America, no. 39 (A. Poole, P. Stettenheim, and F. Gill, Eds.). The Academy of Natural Sciences, Philadelphia, and The American Ornithologists' Union, Washington, DC.

19. Weise, C. M., and J. R. Meyer. 1979. Juvenile dispersal and development of site-fidelity in the Black-capped Chickadee. Auk 96:4–55.

5.8 ACKNOWLEDGMENTS

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Table 5.1 Estimated prevalence of beak deformities in Northwestern Crows captured at six sites in Alaska during winter in 2007 and 2008.

Site	Juveniles		Adults	
	% deformed	*n*	% deformed ± SE	*n*
All sites[a]	0 %	50	16.9 ± 5.3%	135
Seward	0 %	10	33.3 ± 11.1%	18
Kenai	0 %	10	35.7 ± 12.8%	14
Homer	0 %	1	3.2 ± 3.2%	31
Valdez	0 %	9	10.7 ± 5.8%	28
Juneau	0 %	8	14.3 ± 7.6%	21
Haines	0 %	12	4.3 ± 4.3%	23

Table 5.2 Morphometrics[a] of birds with normal beaks compared to those with deformity on upper, lower, or both parts of beak among adult Northwestern Crows captured in Alaska during winter in 2007 and 2008.

		Nares to tip (mm)		Gonys (mm)		Relative overgrowth (mm)	
Status	*n*	Mean ± SE	Min – Max	Mean ± SE	Min – Max	Mean ± SE	Min – Max
Normal							
Males	60	34.6 ± 0.04	29.8 – 39.6	26.5 ± 0.03	22.1 – 31.1	2.2 ± 0.02	0 – 4.5
Females	56	33.6 ± 0.04	28.7 – 40.1	25.6 ± 0.03	20.2 – 30.7	2.3 ± 0.02	0 – 4.9
Upper deformity	11	40.3 ± 0.3	36.9 – 45.8	28.0 ± 0.2	24.7 – 30.8	7.8 ± 0.3	5.5 – 14.1
Lower deformity	4	32.6 ± 1.5	23.9 – 36.8	35.4 ± 1.7	31.5 – 45.5	-8.4 ± 2.8	-25.3 – -0.6
Upper & lower deformity	4	42.3 ± 0.7	39.0 – 45.4	32.3 ± 0.2	31.1 – 33.2	5.4 ± 0.5	3.3 – 8.4

[a] Nares to tip measured from anterior end of right nare to tip of upper beak; gonys measured from central notch on lower beak to tip; relative overgrowth calculated as amount tip of upper beak exceeds (+) or is shorter than (-) tip of lower beak (overbite - underbite; see Supplementary Information).

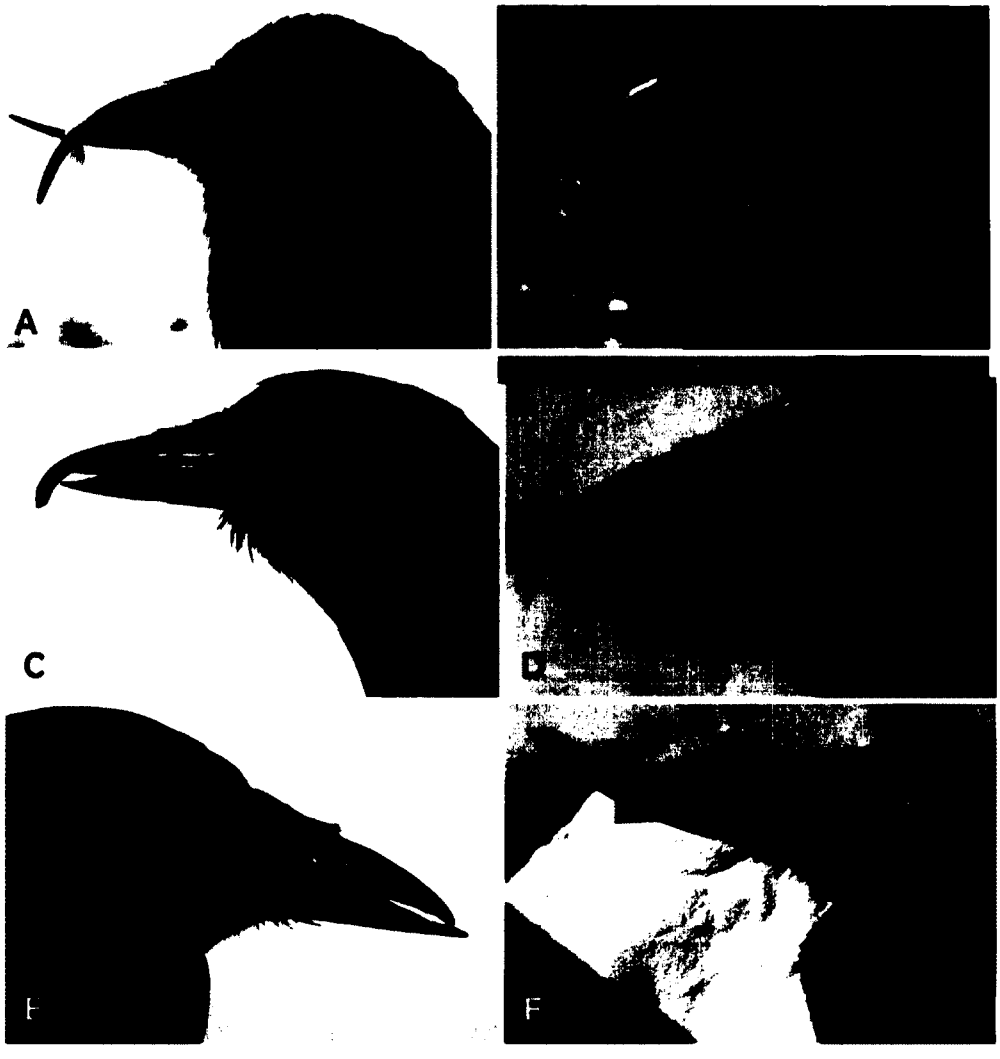

Figure 5.1 Examples of beak deformities in crows in the Pacific Northwest. Severe overgrowth and crossing of upper and lower beaks in crows from A) Juneau, Alaska (photo by Ron Horn), and B) Seward, Alaska (photo by Bill O'Brien). Elongated upper beak with overbite from C) Edmonds, Washington (photo by Kevin Mack), and D) Juneau, Alaska (USGS photo). Elongated lower beak with underbite from E) Valdez, Alaska (USGS photo), and F) Seattle, Washington (photo by John Huckabee). All birds from Alaska are Northwestern Crows; those from Washington could be Northwestern or American crows.

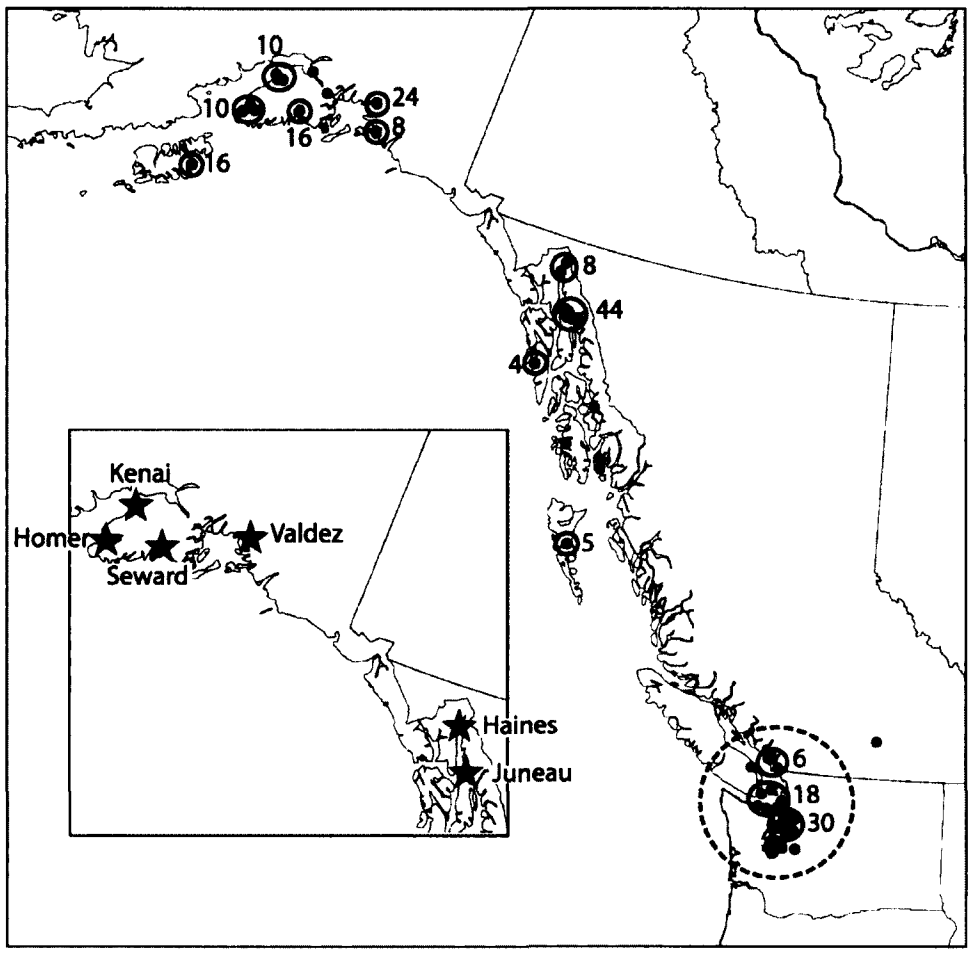

Figure 5.2 Map showing distribution of all documented beak deformities in crows in the Pacific Northwest. Black dots represent individual sightings of Northwestern Crows in Alaska, and of what could be Northwestern or American crows where their distributions overlap in southern British Columbia and northern Washington. Numeric subtotals adjacent to open circles show minimum numbers of individuals with beak deformities for areas where there were multiple observations. Inset map shows study sites (stars) in Alaska where we estimated the prevalence of deformities in Northwestern Crows during winter 2007 and 2008. Most reports from southern British Columbia and northwestern Washington (dashed circle) occurred within a known area of overlap between ranges of Northwestern and American crows.

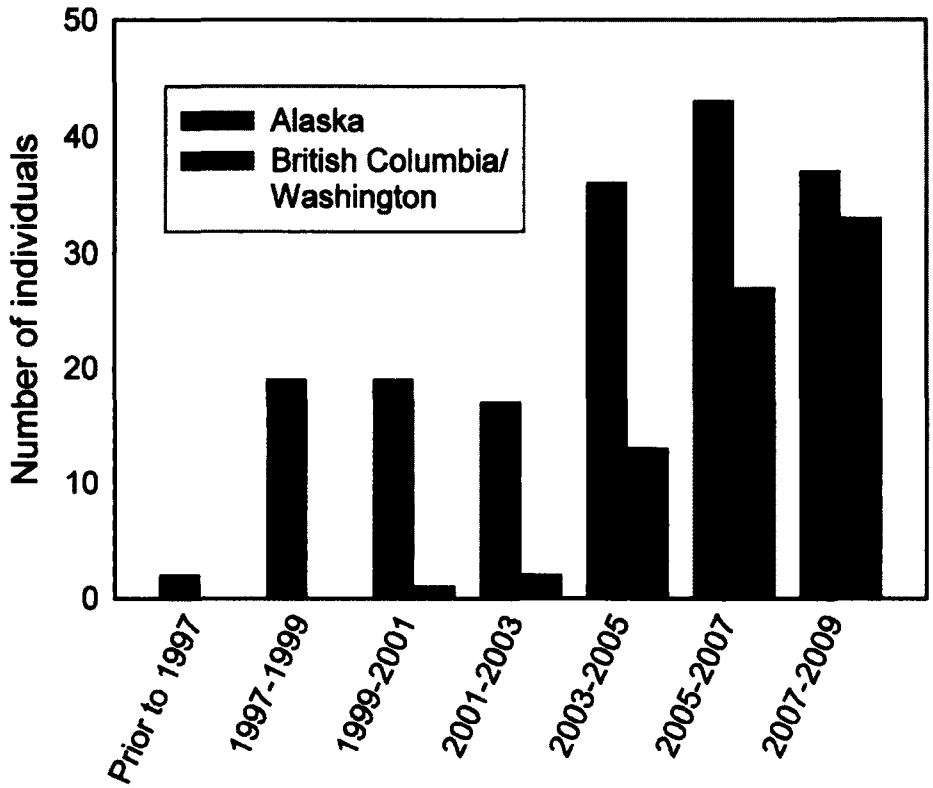

Figure 5.3 Numbers of sightings of crows with beak deformities in crows in the Pacific Northwest between 1980 and 2009. Observations include Northwestern Crows in Alaska (black bars) and Northwestern or American crows in British Columbia and Washington (gray bars). Summaries for each period begin 1 July of the first year listed and are tallied through 30 June of the following year listed.

APPENDIX 5.1: SUPPLEMENTARY INFORMATION

Field methods

Between March 2007 and April 2008, we captured a total of 186 Northwestern Crows at six sites in coastal Alaska using modified drop-net traps (Willson and Comet 1993, Caffrey 2002, C. Van Hemert unpubl. data) and bungee-loaded whoosh nets (Sutherland et al. 2004). For each crow captured, we recorded the following standard measurements: mass, unflattened wing chord, tail length, and diagonal tarsus. We identified birds as juveniles (<1 year of age) or adults (≥1 year) based on molt limit, rectrix shape, and mouth color (Pyle 1997). We used molecular techniques to determine gender from blood samples drawn from the brachial vein (Handel et al. 2006, 2010). All birds were marked with a U. S. Geological Survey (USGS) stainless steel leg band and a unique combination of three plastic colored leg bands for visual identification. Work was completed under guidance of the University of Alaska Fairbanks and the USGS Alaska Science Center Institutional Animal Use and Care committees (Assurance #07-049).

For beak morphometrics, we followed protocols outlined by Handel et al. (2010) and used digital calipers to measure (to 0.1 mm) the chord-length of the upper beak from nares to tip; gonys of the lower beak; any overbite or underbite; and the direction and extent of any lateral crossing of upper and lower beaks. We then assigned a field classification of each beak as normal, deformed, or unknown. Field classifications were based on the expected range of variation assessed from museum specimens measured at the American Museum of Natural History (C. Van Hemert unpubl. data) and historical

live capture data (Johnston 1961, R. Ha unpubl. data). We considered a beak to be deformed if it met any of the following conditions: overbite > 7.5 mm, nares to tip > 45.0 mm, underbite > 3.5 mm, or gonys > 35.0 mm. These criteria generally identified abnormalities that were grossly visible and often detectable from a distance of several meters or more. Individuals that exhibited more subtle beak overgrowth (overbite > 5.0 mm; nares to tip > 40.0 mm; underbite > 0.5 mm; or gonys > 31.0 mm) or possible incipient deformities were field-classified as unknown. All other beaks were classified as normal. We photographed each bird's beak and documented any unusual growth patterns, ridges, or other irregularities. In addition, we examined all keratin structures, including beaks, claws, and skin, for possible abnormalities. Terminology and measurements of the beak follow Lucas and Stettenheim (1972) and Pyle (1997).

Data analysis

To determine the deformity status of beaks that were field-classified as unknown, we used an objective, iterative approach to establish prediction intervals for the range of expected background variation in adult beak morphometrics. This method was based on the rationale that, given the lack of historical background data on beak morphometrics, we could use measurements from the relatively large sample of apparently normal birds in our study (n = 115) to identify prediction intervals beyond which a beak would be considered deformed. These upper and lower bounds could then be used to determine whether a given measurement exceeded the range of normal values for our sample

population. We observed no evidence of beak deformities in juveniles so we restricted all analyses to adult birds.

First, we combined overbite and underbite measurements into a single, continuous variable termed "relative overgrowth," which was a measure of the amount by which the tip of the upper beak exceeded (positive value) or was shorter than (negative value) the tip of the lower beak (calculated as overbite – underbite). We conducted a multiple regression analysis for each of the three beak variables (nares to tip, gonys, and relative overgrowth) as a function of gender and several morphometric indicators of body size (wing length, tail length, tarsus length, and body mass) for adult birds with beaks field-classified as normal. We calculated the standardized residuals ($e_i/\sqrt{MSE}$) and examined normal probability plots to confirm that data met the assumption of Gaussian distribution.

In the first step of the iterative classification process, we used the three resulting equations to estimate 99.5% prediction intervals for sex- and size-adjusted residuals of nares to tip, gonys, and relative overgrowth for the field-classified normal birds. We then used these equations to estimate the residuals for nares to tip, gonys, and relative overgrowth for each individual with a beak field-classified as unknown. Any beaks that fell within the "normal" prediction intervals for a particular measurement were reclassified as normal for that measurement. In the next step, we then included these reclassified individuals in calculations to estimate a new prediction interval. We repeated this process for each of the three beak variables until no new individuals were included. We then used these final prediction intervals for residuals of nares to tip, gonys, and relative overgrowth to classify a beak as normal or deformed (Table 5.S1). Beaks that

fell outside of the prediction intervals for one or more of the three beak morphometrics were classified as deformed. We then used logistic regression (Zelterman 2006) to model the presence or absence of beak deformities within individual adult birds relative to site and gender. We calculated the overall prevalence of deformities across our study areas as the mean of the six site-specific rates. All statistical analyses were conducted in SPSS version 14.0 (SPSS Inc., Chicago, Illinois).

Literature Cited

Caffrey, C. 2002. Catching crows. North American Bird Bander. 26:137–145.

Handel , C. M., L. M. Pajot, S. M. Matsuoka, C. Van Hemert, J. Terenzi, D. M. Mulcahy, C. U. Meteyer, and K. A. Trust. 2010. Epizootic of beak deformities among wild birds in Alaska: An emerging disease in North America? Auk 127:in press.

Handel, C. M., L. M. Pajot, S. L. Talbot, and G. K. Sage. 2006. Use of buccal swabs for sampling DNA from nestling and adult birds. Wildlife Society Bulletin 34:1094–1100.

Johnston, D. W. 1961. The Biosystematics of American Crows. University of Washington Press, Seattle.

Lucas, A. M., and P. R. Stettenheim. 1972. Avian anatomy: Integument. Part I. Agricultural Handbook 362:1–33.

Pyle, P. 1997. Identification Guide to North American Birds: Part 1. Slate Creek Press, Bolinas, California.

Sutherland. W. J., I. Newton, and R. E. Green. 2004. Bird Ecology and Conservation: A Handbook of Techniques. Oxford University Press, New York.

Willson, M. F., and T. A. Comet. 1993. Food choices by Northwestern Crows: Experiments with captive, free-ranging and hand-raised birds. Condor 95:596–615.

Zelterman, D. 2006. Models for Discrete Data. Oxford University Press, New York.

Table 5.S1 Coefficients from multiple regression models of three beak morphometrics (nares to tip, gonys, and relative overgrowth) as functions of sex and measurements of body size (wing, tarsus, tail, and mass) among Northwestern Crows with normal beaks captured during winter in 2007 and 2008 in Alaska. We used an iterative process (see Supplementary Methods) to determine 99.5% iterative prediction intervals (PI) of residuals for normal beak measurements. Beaks whose sex- and size-adjusted residuals were outside the PI were classified as deformed.

	Regression coefficients						99.5% PI of residuals	
Morphometric[a]	Intercept	Sex[b]	Wing (mm)	Tarsus (mm)	Tail (mm)	Mass (g)	Lower	Upper
Nares to tip	-2.074	-0.017	0.028	0.286	0.058	0.014	-5.45	5.88
Gonys	8.989	-0.453	0.038	0.243	-0.065	0.013	-5.20	5.69
Relative overgrowth	0.544	0.213	-0.025	-0.038	0.054	0.005	-2.02	2.12

[a] Nares to tip measured from anterior end of right nare to tip of upper beak; gonys measured from central notch on lower beak to tip; relative overgrowth calculated as amount tip of upper beak exceeds (+) or is less than (-) tip of lower beak (overbite - underbite; see Supplementary Methods).

[b]For variable "sex" used in model, males = 0, females = 1.

CONCLUSIONS

A recent outbreak of beak deformities in Alaska alerted biologists about an emerging disease of wild birds. Avian keratin disorder occurs in multiple, mostly resident species and at extremely high prevalence in affected populations (6.5% in Black-capped Chickadees, 16.9% in Northwestern Crows; Handel et al. 2010; Chapter 5). This disorder is characterized by overgrowth of the rhamphotheca, the outer keratinized layer of the beak, and results in gross elongation and crossing (Chapters 1, 3). The presentation of beak deformities in chickadees and other affected species is not consistent with any other known avian diseases, suggesting the emergence of a novel disorder in Alaskan birds (Chapter 3).

The first part of my dissertation research addressed the physiological, microscopic, and ultrastructural changes associated with avian keratin disorder in Black-capped Chickadees. The underlying mechanisms and cellular abnormalities associated with macroscopic changes in the beak had not yet been characterized and presented a significant knowledge gap in our understanding of this disorder. I approached this research need by concurrently studying the physical mechanisms by which deformities arise and searching for microscopic clues within affected animals. In a captive study of Black-capped Chickadees, I found that rates of beak growth were 50-100% higher and much more variable in affected birds than unaffected birds (Chapter 1). These results demonstrate that hyperplastic growth is the primary physical mechanism by which deformities develop. In addition, differential rates of beak wear influenced the severity

of deformities, which has important implications for beak function. Repeated breakage of beak tips significantly mitigated the effects of rapid growth in some individuals, resulting temporarily in a more normal appearance of the beak. This pattern may partially explain the persistence of some affected animals in the wild despite apparently debilitating beak overgrowth (Chapter 1; Handel et al. 2010). However, I also observed a mortality rate of more than 50% and high occurrence of skin lesions among captive birds with beak deformities, demonstrating severe health consequences of this disorder (Chapters 1, 3).

Histology reveals microscopic anatomy of cells and tissues and is used as a diagnostic tool, often in combination with other techniques such as electron microscopy. In human and veterinary medicine, comparison between abnormal and normal tissues is often the first step in identifying the cause of a tumor or lesion. However, to characterize the pathology of avian keratin disorder in this study, I first needed to establish baseline information about normal beaks and claws in Black-capped Chickadees. Despite the importance of these structures, few efforts have been made to describe their internal anatomy. To help address this research gap, Chapter 2 depicts functional microanatomy of passerine beaks and claws using modern imaging techniques. As part of this effort, I also developed methods for processing hard-cornified tissues, which provides an important resource for study of avian keratin disorder and other diseases of the beak.

I used a combination of diagnostic tools to identify cellular and tissue abnormalities and screen for potential etiological agents in affected birds. Radiography, histopathology, and electron microscopy revealed characteristic microscopic and

ultrastructural features associated with avian keratin disorder (Chapter 3). The primary lesions in the beak included hyperkeratosis, hyperplasia, and core-like intrusions of necrotic debris along the junctional line of the inner and outer cornified plates. I also observed apparent structural abnormalities in the cornified tissue of the beak. Interestingly, claws exhibited similar pathology, despite their less conspicuous macroscopic presentation. These results are consistent with hyperplastic epidermal growth and demonstrate that multiple keratinized tissues are affected, thus indicating a generalized disorder. Skin, feathers, and sinuses also exhibited hyperkeratosis, but only in the presence of microbial pathogens, suggesting that these are likely to be secondary consequences of avian keratin disorder. Such changes could be due to reduced preening ability resulting from beak overgrowth or related to underlying systemic pathology, such as compromised immune function.

Although the specific etiology of avian keratin disorder is not yet known, my research on the physiology and pathology of beak deformities has provided valuable insights about possible mechanisms and causative agents. We now understand that normal processes of keratin production have been disrupted in affected birds, resulting in accelerated epidermal growth. The detailed characterization of gross and microscopic changes has also allowed me to rule out a number of likely etiologies. Chickadees with beak deformities did not display pathology that is consistent with any reported nutritional problems (Klasing 1998; Keymer and Samour 2008). In particular, deficiencies of Vitamin A, zinc, or biotin can cause beak overgrowth and abnormalities in keratinized tissues, but such lesions differ in appearance from those observed in chickadees.

Similarly, beak abnormalities previously attributed to toxic etiologies in other avian species, including exposure to elevated levels of selenium or polychlorinated biphenyls (Ohlendorf et al. 1986; Gilbertson et al. 1991; Ludwig et al. 1996), exhibit distinctive pathology from avian keratin disorder. In addition, I found no evidence of lesions in the liver or other organs commonly affected by some toxicants. Microbial pathogens or parasites, which are normally detectable with the diagnostic methods I used, do not appear to play a primary role in the beak overgrowth characteristic of avian keratin disorder. My research has helped to shorten the list of differential diagnoses and provides guidance for upcoming research.

I have identified several avenues for future investigation of the physiology and pathology of avian keratin disorder. First, additional study is needed to determine the specific mechanisms responsible for abnormal keratin production in affected birds. Biochemical analysis, immunohistochemistry, and targeted genomic analysis of beak and claw tissues would provide valuable insights about keratin expression and pathogenesis. Such efforts would help to identify individual keratins that may be disrupted and could potentially serve as biomarkers in affected tissues. Specific diagnostic testing is also needed to identify the underlying cause(s) of the disorder. Results from Chapter 3 suggest that a viral etiology is plausible. Targeted electron microscopy work to determine whether viral particles are present in the germinal epithelium of beak and claw tissue from affected birds would help to resolve this issue. Additional toxicological analysis to rule out environmental contaminants as etiological agents of this disorder is also necessary. Dioxins and dioxin-like compounds have been implicated in keratin

abnormalities in other taxa (Vos and Beems 1971; Jackson and Halbert 1974; Kimbrough 1984; White and Birnbaum 2009), and could potentially contribute to the development of beak deformities. Other naturally-produced toxins, including mycotoxins, have not yet been investigated and should also be considered in future study. Finally, a better understanding of the underlying controls of beak growth would help to identify factors involved in avian keratin disorder.

The other major component of my Ph.D. research used field studies to investigate ecological issues related to beak deformities in wild birds. I focused on two outstanding questions: 1) Is there an association between diet and beak deformities? 2) Does avian keratin disorder affect multiple species?

Study of diet was relevant for my research because of its potential to play a key ecological role as a cause or consequence of beak deformities. I was particularly interested in whether affected birds consumed a larger proportion of anthropogenic foods, such as those provided in bird feeders. To address this issue, I used stable isotope analysis to characterize winter dietary composition in chickadees. I found that diets differed between Black-capped Chickadees with normal beaks and beak deformities, but that such differences were more likely a result than a cause of avian keratin disorder (Chapter 4). Affected birds generally consumed fewer arthropods and sunflower seeds and more peanut butter and natural seeds and berries, a pattern that is likely related to reduced function of the beak. Although some individuals with beak deformities relied heavily on feeder foods, we did not find evidence of an anthropogenic diet source shared by all affected birds. However, changes in diet due to reduced function of the beak may

have important nutritional implications, including limiting protein sources, and could contribute to compromised fitness associated with beak deformities in chickadees (Handel et al. 2006; Handel et al. 2010).

Study of avian keratin disorder in Alaska has demonstrated significant consequences for individual health and fitness (Handel et al. 2010; D'Alba et al. 2011). Gross overgrowth of the beak seriously compromises birds' ability to feed, preen, and cache foods, all of which are necessary for thermoregulation and survival in northern climates. Changes in dietary composition have important implications for avian health and nutrition, especially given the high prevalence of this disorder in multiple species and across a growing geographic area (Handel et al. 2010; Chapter 5). In our field studies of this disorder, we often observed birds with beak deformities exhibiting signs of poor health, including dirty, matted plumage and mild to severe emaciation (Handel et al. 2006; Handel et al. 2010). Evidence of high mortality rates (Chapter 1) and reduced reproductive success (Handel et al. 2006; Handel et al. 2010) indicate that this condition has the potential to significantly impact avian populations. Upcoming analysis of long-term banding data will allow us to estimate survival rates in the wild and will provide additional insights about the population-level effects of avian keratin disorder.

Although the disorder was first documented in Black-capped Chickadees, morphologically similar deformities subsequently appeared in other species throughout Alaska and the Pacific Northwest. My research on Northwestern Crows confirmed high prevalence of a nearly identical condition to that observed in chickadees, indicating that

avian keratin disorder affects multiple, ecologically-distinct species (Chapter 5). Observations of crows with beak deformities were also documented across a larger geographic area than originally reported for chickadees, including much of Alaska and the Pacific Northwest. Recent evidence suggests that the disease may be further spreading beyond this region. Similar clusters of deformities have subsequently been reported in other parts of North America and the United Kingdom (Harrison 2011).

The rapid emergence of avian keratin disorder over the past decade suggests a relatively recent ecological or environmental change. Interestingly, although affected species identified within each region are taxonomically and ecologically diverse, similar patterns have been observed across different regions (Chapter 5; Handel et al. 2010; Harrison 2011). Species most commonly affected include members of the families Paridae (chickadees, tits) and Corvidae (crows, rooks, ravens, magpies, jays). Woodpeckers (Picidae) and nuthatches (Sittidae) have also been frequently reported. The apparent occurrence of a parallel disorder in geographically disparate areas highlights the need to identify epidemiological patterns and movements of potential pathogens.

Additional studies are currently underway to determine the causes and consequences of this puzzling disorder. Results from my Ph.D. work provide an important scientific foundation and have helped to identify priority areas for future research on avian keratin disorder.

Literature cited

Craves, J. A. 1994. Passerines with deformed bills. North American Bird Bander 19:14-18.

D'Alba, L., C. Van Hemert, C. M. Handel, and M. D. Shawkey. 2011. A natural experiment on the condition-dependence of achromatic plumage reflectance in Black-capped Chickadees. PLoS ONE 6:e25877.

Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2001. Infectious disease and amphibian population declines. Diversity and Distributions 9:141-150.

Dobson, A. P., and J. Foufopoulos. 2001. Emerging infectious pathogens of wildlife. Philosophical Transactions of the Royal Society of London B Biological Sciences 356:1001-1012.

Gilbertson, M., T. Kubiak, J. Ludwig, and G. Fox. 1991. Great Lakes embryo mortality, edema, and deformities syndrome (GLEMEDS) in colonial fish-eating birds: similarity to chick-edema disease. Journal of Toxicology and Environmental Health 33:455-520.

Handel, C. M., L. M. Pajot, S. M. Matsuoka, K. A. Trust, J. M. Stotts, J. Terenzi, and S. L. Talbot. 2006. Potential role of environmental contaminants in the pathology of beak deformities among Black-capped Chickadees in south-central Alaska. USGS Alaska Science Center, Anchorage, Alaska.

Handel, C. M., L. M. Pajot, S. M. Matsuoka, C. Van Hemert, J. Terenzi, S. L. Talbot, D. M. Mulcahy, C. U. Meteyer, and K. A. Trust. 2010. Epizootic of beak deformities

among wild birds in Alaska: an emerging disease in North America? Auk 127:882-898.

Harrison, G. J. 1986. Disorders of the integument. Pages 397-407 in Clinical Avian Medicine and Surgery (G. J. Harrison, and L. R. Harrison, Eds.). W. B. Saunders Co., Philadelphia, Pennsylvania.

Harrison, T. 2011. Beak deformities of garden birds. British Birds 104:538-541.

Jackson, T., and F. Halbert. 1974. A toxic syndrome associated with the feeding of polybrominated biphenyl-contaminated protein concentrate to dairy cattle. Journal of the American Veterinary Medical Association 165:437-439.

Johnson, P. T. J., J. M. Chase, K. L. Dosch, R. B. Hartson, J. A. Gross, D. J. Larson, D. R. Sutherland, and S. R. Carpenter. 2007. Aquatic eutrophication promotes pathogenic infection in amphibians. Proceedings of the National Academy of Sciences 104:15781-15786.

Keymer, I. F., and J. Samour. 2008. Disorders of the digestive system. Pages 281-302 in Avian Medicine (J. Samour, Ed.). Elsevier, New York.

Kimbrough, R. D. 1984. Skin lesions in animals and humans: a brief overview. Banbury Report 18:357-363.

Klasing, K. C. 1998. Comparative Avian Nutrition. Cab International, New York.

Ludwig, J. P., H. Kurita Matsuba, H. J. Auman, M. E. Ludwig, C. L. Summer, J. P. Giesy, D. E. Tillitt, and P. D. Jones. 1996. Deformities, PCBs, and TCDD-equivalents in Double-crested Cormorants (*Phalacrocorax auritus*) and Caspian

Terns (*Hydroprogne caspia*) of the upper Great Lakes 1986-1991: testing a cause-effect hypothesis. Journal of Great Lakes Research 22:172-197.

Ohlendorf, H. M., D. J. Hoffman, M. K. Saiki, and T. W. Aldrich. 1986. Embryonic mortality and abnormalities of aquatic birds: apparent impacts of selenium from irrigation drainwater. Science of the Total Environment 52:49-63.

Pomeroy, D. E. 1962. Birds with abnormal bills. British Birds 56:49-72.

Stettenheim, P. R. 2000. The integumentary morphology of modern birds - an overview. American Zoologist 40:461-477.

Tully, T. N., Jr., M. P. C. Lawton, and G. M. Dorrestein, Eds. 2000. Handbook of Avian Medicine, 2nd ed. Butterworth-Heinemann, Elsevier Science Ltd., New York.

Vos, J. G., and R. B. Beems. 1971. Dermal toxicity studies of technical polychlorinated biphenyls and fractions thereof in rabbits. Toxicology and Applied Pharmacology 19:617-633.

White, S. S., and L. S. Birnbaum. 2009. An overview of the effects of dioxins and dioxin-like compounds on vertebrates, as documented in human and ecological epidemiology. Journal of Environmental Science and Health, Part C: Environmental Carcinogenesis and Ecotoxicology Reviews 27:197-211.