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Refinement of biomarker pentosidine methodology for use on aging birds

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***Abstract*:** There is no reliable method for determining age for most species of long-lived birds. Recent success using the skin chemical pentosidine as a biomarker has shown promise as an aging tool for birds. Pentosidine levels have been determined only from the breast tissue of carcasses, and we sought to refine the procedure with respect to biopsy size and location for safe and effective use on living birds. We compared pentosidine concentrations in 4 skin-size samples (4, 6, 8, and 20-mm diameter biopsies) from the breast of black vulture (*Coragyps atratus*) carcasses. We also compared pentosidine levels from breast and patagial tissue to document potential differences among collection sites of deceased vultures (with unknown ages) and monk parakeets (*Myiopsitta monachus*; with actual, minimal, and unknown ages). Pentosidine concentrations (pmol pentosidine/mg collagen) were similar among the 4 sizes of vulture breast skin ($P = 0.82$). Pentosidine concentrations for the breast ($\bar{x} = 8.9$, SE = 0.55, $n = 28$) and patagium ($\bar{x} = 8.9$, SE = 0.51, $n = 28$) of vultures were similar, but in parakeets, pentosidine was higher in the breast ($\bar{x} = 15.9$, SE = 1.30, $n = 105$) than the patagium ($\bar{x} = 11.5$, SE = 1.10, $n = 105$). We made pentosidine-based age estimates for vultures and parakeets using a general age curve for wild birds. We also made vulture age estimates using plumage characteristics and a cormorant (*Phalacrocorax auritus*) age curve. Vulture pentosidine-based age estimates appear to correspond to plumage-based age estimates. Pentosidine-based age estimates for 88% of the known-aged parakeets ($n = 17$) were within 6 months of actual ages. Even though known ages were not available for all birds, we found a positive trend in pentosidine versus age for both species. We suggest that 6-mm diameter skin samples from the patagium of living vultures and other similar-sized birds will provide sufficient tissue for reliable age estimation and will not impair flight ability.

Key words: age, biomarker, black vulture, *Coragyps atratus*, human–wildlife conflicts, monk parakeet, *Myiopsitta monachus*, patagium, pentosidine, pest species, skin

ONCE A PEST SPECIES EXCEEDS societal acceptance capacity, management may be initiated to control or reduce damages or other nuisance activities. Wildlife damage management often incorporates lethal (Humphrey et al. 2004) or reproductive control measures (Yoder et al. 2007, Avery et al. 2008). With birds, age estimates prove useful in developing life tables, pre-management model simulations, modeling to determine how many of a species need to be euthanized or sterilized to maintain population levels within social acceptance capacities, and projecting population response to management techniques (Dolbeer 1998). The accuracy of these models will increase with the availability of age-specific survival and fecundity, and age distribution data (Blackwell et al. 2007).

Using pentosidine aging research for birds may be the catalyst for discovering more effective management strategies for pest and nonindigenous species. For this study, we used black vultures (*Coragyps atratus;* hereafter, vultures) and monk parakeets

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(*Myiopsitta monachus*; hereafter, parakeets) in the refinement of the pentosidine assay technique for birds because specimens are available and both birds are pest species of concern (Lowney 1999, Strafford 2003). A viable pentosidine aging technique could be used to acquire an understanding of the biology of species of interest as a necessary precursor to the development of efficient and effective wildlife damage management and conservation strategies.

Bird-banding studies often take a long time to acquire useable age-structure data, unlike pentosidine, which has the potential to determine the age structure of a small population in a matter of months. Pentosidine is a product of the Maillard or browning reaction, resulting from the non-enzymatic glycosylation of collagen (Sell and Monnier 1989). It is a stable (Monnier 1989), fluorescent (Sell et al. 1998), and irreversible collagen crosslink (Sell and Monnier 1989). Pentosidine is found in many different tissues and organs (e.g., skin), and it accumulates throughout the lifetime of the individual, which makes it a useful biomarker for chronological age (Monnier et al. 1993). Pentosidine analysis has been validated as a reliable method of age estimation in numerous bird species, such as domestic poultry (Iqbal et al. 1997), parrots (*Ara* spp. and *Cacatua moluccensis*), and bald eagles (*Haliaeetus leucocephalus*; J. A. Fallon, National Aviary, and C. K. Cooey, West Virginia University, unpublished data), ruffed grouse (*Bonasa umbellus*; Fallon et al. 2006*a*, *b*), double-crested cormorants (*Phalacrocorax auritus*; Fallon et al. 2006*a*, Cooey 2008), and various species of wild birds (Chaney et al. 2003).

Most of the previous pentosidine aging studies have used breast skin from deceased birds, but to realize the full potential of this method, adaptations to sample living birds are desirable. Such techniques must consider both the health and welfare of the bird and the technical feasibility of acquiring sufficient tissue for analysis. Because the breast contains the major flight muscles for birds, sampling skin from the breast could seriously impair their flying ability. Marking birds with patagial tags has been a standard technique for many years, and several post-marking monitoring studies indicate that many birds suffer few deleterious effects from these tags (Marion and Shamis 1977, Wallace et al. 1980, Sweeney et al. 1985). However, several studies, including Southern and Southern (1985) and Calvo and Furness (1992), indicate that patagial tags do negatively affect birds, so care must be taken when obtaining skin biopsies. The patagium also contains fewer veins than the breast (Proctor and Lynch 1993), thus, decreasing the chance of an infection to develop (Muza et al. 2000). The patagium, therefore, seems like a suitable location for obtaining skin samples from live birds.

Fallon et al. (2006*b*) found that pentosidine in ruffed grouse ($n = 6$) was higher in the patagium compared to the breast. They speculated that this finding may be due to differences in vascularization, relative body temperature, rates of collagen turnover, or concentrations of tissue antioxidants in various locations of a bird's body. Thus, our first objective was to compare pentosidine from breast and patagial skin samples to determine if age curves need to be created for different areas of the body for birds. This study will help determine if, for example, an age curve developed entirely from patagial skin could be used to provide an accurate age estimate for a breast skin sample. We predict that the concentration of pentosidine will be different between the patagium and breast, and age curves will need to be developed for both areas of the body. Further, skin samples analyzed from dead birds in previous studies were approximately 20 mm in diameter (Chaney et al. 2003; Fallon et al. 2006*a*, *b*), which is not feasible for use when sampling living birds. Our second objective, therefore, was to determine the minimum size required for accurate pentosidine measurement. We predict that there will be no difference in pentosidine concentrations between all skin sample sizes.

Study species

Vultures are long-lived birds with a potential life span in excess of 20 years (Buckley 1999). Vultures are considered pests because of the damage they do to homes and businesses from roosting (Fitzwater 1988), colliding with aircraft (Dolbeer et al. 2000, DeVault et al. 2005), and depredating livestock and poultry (Avery and Cummings 2004). Population age structures

and key aspects of their life history, such as age of first breeding (Parker et al. 1995) remain unknown, because these birds cannot be aged reliably (Blackwell et al. 2007).

Parakeets are small, omnivorous birds that were introduced to the United States from South America via the pet trade in the 1960s (Long 1981, Russello et al. 2008). Increasing population sizes (van Bael and Pruett-Jones 1996), potential to spread Newcastle disease (Fitzwater 1988), and damage resulting from building nests on utility poles, transmission line support towers, and electric substations (Avery et al. 2002, Tillman et al. 2004) have given this species a reputation as a pest. Banding studies in parakeets' native range indicate a potential lifespan of at least 6 years in the wild, but age structures of invasive populations in the United States are unknown (Spreyer and Bucher 1998).

We chose to work with vultures and parakeets because of the need to learn more about age classes of wild populations for improved management of them. Having a better understanding of age-specific life-cycle parameters, such as survival rates and reproductive success, can help in predicting how populations will respond to different forms of management (Tuljapurkar and Caswell 1997). Thus, for both of these species, development of a verifiable age estimation method is warranted. The preserved carcasses for both species obtained by USDA/APHIS/Wildlife Services (WS) provided the required amount of skin needed to refine the pentosidine aging technique.

Methods

Sample collection

In May 2004, we collected 1 vulture as a roadkill, and we live-trapped 29 vultures as part of a vulture population-management program in Gainesville, Florida. Vultures were euthanized using carbon dioxide, as described by Beaver (2001). We collected approximately 150-mg skin samples from the breast of the vultures at necropsy for use in the skin-size study, froze samples in distilled water, and mailed them overnight for analysis to West Virginia University (WVU), Morgantown, West Virginia, in 2004. We retained the frozen carcasses at WS' National Wildlife Research Center (NWRC) field station in Gainesville, Florida. In December 2006, we thawed the carcasses and collected patagial skin samples using a 6-mm diameter Sklar Tru-Punch disposable biopsy punch (Sklar, West Chester, Penn.) to compare pentosidine concentrations in the breast and patagium. We froze and mailed the samples overnight to WVU for analysis. Advanced glycation endproducts have a half-life of 117 years in cartilage collagen and 15 years in skin collagen of humans (Verzijl et al. 2000). Collagen has a triple helical structure with strong inter- and intra-molecular bonds (Freifelder 1983), and hydrocarbon chains of several amino acids form tight hydrophobic clusters, resulting in an organic compound that could exist indefinitely if stored in dry environments (Aufderheide 1981). Collagen in ruffed grouse skin was found to remain stable while frozen at ≤4°C from September 2006 ($\bar{x}$ = 0.455 mg, SE = 0.048, n = 9) to February 2010 ($\bar{x}$ = 0.396 mg, SE = 0.057, n = 9) (P = 0.42; C. K. Cooey, West Virginia University, unpublished data). Based on this information and findings in museum study skins that pentosidine remained stable for at least 1 year from the time of the birds' death (Fallon et al. 2006*b*), we assumed that pentosidine in our samples remained stable.

From 2002 to 2007, we live-trapped 105 parakeets from wild populations in Miami-Dade County, Florida. We used long-handled nets to capture the birds as they flew out of their nests (Martella et al. 1987). We euthanized some (n = 64) of the birds using carbon dioxide gas (Gaunt and Oring 1999) and held some in captivity (n = 41) at the NWRC field station in Gainesville, Florida. Those held in captivity either died naturally or were later euthanized using carbon dioxide gas. Seventeen of the captive birds had known ages because they were captured as juveniles (age range 1 to 18 months), while the remaining 24 birds were captured as adults and held in captivity for 2 to 50 months, where they had at a minimum age (range 24 to 60 months old).

We froze euthanized parakeets (n = 97) for 5 to 50 months before collecting skin samples. In January 2007, we allowed the preserved parakeets to thaw for 30 to 60 minutes and euthanized the live parakeets (n = 8) before we collected samples. We removed approximately 50 mg of skin from the breast (as well as the

entire patagium from the left wing) from each parakeet and froze the samples until analysis.

Laboratory analysis

We processed all skin samples within 2 to 3 months of collection. Repeated freezing and thawing have shown no influence on pentosidine concentrations. We analyzed the breast samples of the vultures in 2004. We compared pentosidine concentrations in 4-, 6-, 8-, and 20-mm-diameter skin samples for each vulture. In 2007, we compared pentosidine concentrations from 6-mm diameter patagial skin samples to the initial pentosidine concentrations from the 6-mm diameter breast skin samples only. We did not have enough skin from the parakeets to evaluate differences among sizes, so we processed 20-mm diameter skin samples (approximately 40 mg, standard processing size) to determine if differences exist between pentosidine concentrations in breast and patagial sampling sites.

We prepared all vulture and parakeet skin samples for pentosidine determination using a modified Iqbal et al. (1997) technique. Briefly, this process involved skin preparation (removal of adipose tissue and subdermal layers and mincing), delipidation (5 ml of 2:1 chloroform:methanol solution for 18 hours on an agitator in a 4° C cold room), rehydration (2 to 3 ml of 1:1 methanol:distilled water solution for 2 hours at 20 °C), acid hydrolysis (1 ml of nitrogen flushed 6N HCl per 10 mg skin incubated 18 hours at 110°C), acid evaporation using a Speed-Vac centrifuge dryer (Savant Instruments, Farmingdale, N.Y.) set at continuous run high temperature, a second rehydration (500 µl distilled water), and filtering (using a .45 micron Costar Spin-X centrifuge tube filter (Corning Costar Corp., Cambridge, Mass.) and an Eppendorf 5415 microcentrofuge (Eppendorf, Hauppauge, New York) set at 4,000 rpm for 10 minutes. We determined collagen content through spectrophotometric hydroxyproline analysis using a DU 640 spectrophotometer (Beckman Coulter, Fullerton, Calif.) with a 564 wavelength, assuming 14% of collagen to be hydroxyproline (Maekawa et al. 1970). We measured pentosidine concentrations through reverse-phase high-performance liquid chromatography (HPLC). We analyzed pentosidine samples in duplicate, where 1 sample was spiked with a pentosidine standard to determine elution time. Integration of peaks was done with Millennium 32, version 3.05.01 software (Waters Corporation, Milford, Mass.), later upgraded to Empower 2 software (Waters Corporation, Milford, Mass.).

Bird age estimates

One of the major issues in using the pentosidine aging technique is finding a large enough sample of known-aged birds that span the entire lifespan of each study species. We were limited in not having any known-aged vultures and having only young known-aged parakeets. Because of this, we could not create species-specific age curves for vultures or parakeets. We used age curves that were developed in past studies to provide an estimate of age for vultures and parakeets. We used our limited information about the ages of the vultures (plumage based) and parakeets (captive time and band records) to determine the accuracy of the age estimates from these curves.

A species-specific age curve that uses pentosidine already has been developed using breast skin for double-crested cormorants ranging in age from 6 months to 14.5 years (Fallon et al. 2006*a*). We believe that this will be a suitable age curve to use to estimate vulture ages (because of the similarities between the species) until a vulture-specific age curve is created. Double-crested cormorants are comparable in size (69 cm long, with a 127-cm wingspan; Robbins et al. 1966) to the size of vultures (60 to 68 cm long and 137 to 150 cm wingspan; Buckley 1999). Cormorants also have approximately the same maximum life span (22 years, 6 months; Lutmerding and Love 2009) as vultures (25 years, 6 months; Clapp et al. 1982). Also, the male vultures in this study had mass that averaged 2,087 g, while the females averaged 2,128 g, similar to the average mass of cormorants (1,200 to 2,500 g; Hatch and Wesloh 1999). The cormorant age curve has the logistic equation: $y = 0.1914x + 6.6701$ ($r^2 = 0.93$), in which y = pentosidine concentration and x = estimated age in months (Fallon et al. 2006*a*). In addition, we estimated vulture ages using the general wild-bird curve: $y = 0.2047x + 7.4725$ ($r^2 = 0.73$) (Chaney et al. 2003). This curve was created using skin samples from 29 species of birds ranging in size from a red siskin (*Cardelis*

cucullata) to a great blue heron (*Ardea Herodias*) and in age from a few days to 18.5 years (Chaney 2001). We calculated age estimates for the breast data and the patagial data separately.

We documented external characteristics of each of the vultures to categorize each as a juvenile, sub-adult, or adult. This age estimate was based on the feathering and wrinkles on the head and color of the head and beak (Jackson 1988, Buckley 1999). Juveniles (<1 year; Brown and Amadon 1968, Jackson 1988) were easy to discern at the time of collection because they have black beaks without a pale tip, black skin on their head and neck that lack wrinkles (Jackson 1988, Buckley 1999), sparse, short, bristle-like down on their head and upper neck, and contour feathers that extend farther up the back of the neck (Jackson 1988). Juvenile vultures collected in this study were thought to be only a couple months old (hatch from mid-March to mid-May [Buckley 1999]; collected in May 2004). Adults (>2 years) have deeply furrowed, gray skin on their heads and necks, dark gray beaks with an ivory tip (Buckley 1999), and a bare neck (except for the nape) and head (Jackson 1988). We classified sub-adult vultures (1 to 2 years) as having characteristics between juveniles and adults, such as the increased amount of wrinkling and transitioning coloration of skin on the head, which progresses from black to gray with increasing age (Jackson 1988). We compared our general visual age estimates to those determined from the age curves.

We determined age estimates for parakeets using the wild-bird age curve only (Chaney et al. 2003). We believe that this will be the best curve to use to estimate parakeet age until a parakeet-specific curve is developed because various species of parrots were used in its creation (e.g., *Anodorhynchus hyacinthinus*, *Trichoglossus goldiei*, and *Loriculus galgulus*; Chaney 2001). We compared the estimated ages for the parakeets to the known and minimum ages for these birds to determine the accuracy of the estimated ages

Statistical analysis

We conducted a randomized complete block analysis of vultures (n = 30) using Statistical Analysis Software (SAS) version 9.1 (SAS Institute, Cary, N.C.) to determine if differences exist between pentosidine concentrations of the 4 different skin-sizes. The individual birds were the blocks, the skin size was the treatment, and the pentosidine concentration was the response variable. We set statistical significance at α = 0.05.

We ran paired t-tests (n = 28 [vultures; 2 outliers removed]; n = 105 [parakeets]) with SAS to determine if there were any significant differences in pentosidine concentrations between the breast and patagium. Our dependent variable was the pentosidine concentration, and the independent variable was the body location. We tested data for normality by evaluating skewness (g_1; -1 to +1 range; SAS Institute 2004) and kurtosis (g_2; -3 to +3 range; Newell and Hancock 1984) (g_1 = -0.22, g2 = -0.53[vultures]; g_1 = -0.17, g_2 = 2.86 [parakeets]) and homogeneity of variances by Bartlett's test for homogeneity (χ^2_1 = 0.17, P = 0.68 [vultures] χ^2_1 = 2.87, P = 0.09 [parakeets]). Data met these 2 assumptions, so we did not transform data.

We also ran paired t-tests (n = 28 [vultures; 2 outliers removed]; n = 105 [parakeets]) to determine if the estimated ages for vultures and parakeets, not just pentosidine concentrations, differed between the locations, as well. Our dependent variable was the value for the age, and the independent variable was the location. We tested data for normality by evaluating box plots (g_1 = 0.25, g_2 = -0.56 [cormorant curve for vultures]; g_1 = 0.22, g_2 = -0.51 [wild bird curve for vultures]; g_1 = -0.17, g_2 = 2.87 [parakeets]) and homogeneity of variances by Bartlett's test for homogeneity (χ^2_1 = 0.17, P = 0.68 [cormorant curve for vultures]; χ21 = 0.17 [wild bird curve for vultures], P = 0.68; χ^2_1 = 2.87, P = 0.09 [parakeets]). Data met these 2 assumptions, so we did not transform data.

We ran simple linear regression analysis to compare pentosidine accumulation with age. We used age-class estimates based on plumage characteristics for the vultures and combined known and minimal parakeet ages for the regressions. We ran regressions for the locations separately and for combined data.

Results

Black vultures

Skin-size comparison. The measured pentosidine concentration (pmol Ps/mg collagen) did not vary among 4-mm ($\bar{x}$ = 9.54, SE = 0.66),

6-mm ($\bar{x}$ = 9.71, SE = 0.94), 8-mm ($\bar{x}$ = 9.77, SE = 0.67), and 20-mm ($\bar{x}$ = 9.99, SE = 0.62) skin samples among the 30 vultures ($F_{3,116}$ = 0.27, P = 0.85).

Body location comparison. Vulture pentosidine concentration (n = 28) was similar between breast ($\bar{x}$ = 8.9, SE = 0.55) and patagial ($\bar{x}$ = 8.9, SE = 0.51) skin samples (t_{27} = 0.04, P = 0.97). Using the cormorant curve, age estimates, in months, between breast ($\bar{x}$ = 11.6, SE = 2.85) and patagial ($\bar{x}$ = 11.9, SE = 2.64) skins did not differ for individual vultures (t_{27} = 0.09, P = 0.93). The breast ($\bar{x}$ = 7.0, SE = 2.70) and patagial ($\bar{x}$ = 7.1, SE = 2.49) skins produced similar estimated ages when using the wild-bird curve (t_{27}= -0.04, P = 0.97). Sixty-three percent and 53% of the age estimates using the cormorant and wild-bird curves, respectively, were within 6 months, and 70 and 67%, respectively, were within 1 year of the age-class estimated, using physical characteristics.

Monk parakeets

Pentosidine concentrations varied between the breast ($\bar{x}$ = 15.9, SE = 1.30) and patagium ($\bar{x}$ = 11.5, SE = 1.10) for parakeets (t_{104} = -5.14, P < 0.001, n = 105). Age estimates differed between breast ($\bar{x}$ = 41.0 months, SE = 6.34) and patagial ($\bar{x}$ = 19.8 months, SE = 5.37) skins for individual parakeets (t_{104} = -5.16, P < 0.001, n = 105).

Using the breast skin data, 16 (94%) of the known-age birds had age estimates within 6 months of their actual age and 100% of the birds of known minimum age had age estimates higher than the length of time they spent in captivity. With the patagial skin data, 15 (88%) of the known-age birds had age estimates within 6 months of their actual age, and 22 (92%) of the birds of known minimum age had age estimates higher than captive holding time. The other 2 birds had age estimates only 2 and 12 months lower than their captive holding time.

Pentosidine concentration was found to accumulate with age (actual and minimal; Figure 1). This was seen for both the breast (y = 0.4986x + 4.7053, r^2 = 0.6989, t_{40} = 9.52, P < 0.001) and patagial skins (y = 0.4581x + 3.8609, r^2 = 0.6701, t_{40} = 8.90, P < 0.001), separately, and when both locations were combined (y = 0.4783x + 4.2831, r^2 = 0.6803, t_{81} = 13.05, P < 0.001).

Discussion

Results from this study will be used in the refinement of the pentosidine aging technique with birds. The parakeet study produced similar results to the Fallon et al. (2006*b*) findings for pentosidine differences, although in the parakeet study pentosidine was higher in the breast. These data also suggest that known-age curves for parakeets will need to be developed for 1 specific sampling site. One explanation why pentosidine was higher in the patagium of grouse (Fallon et al. 2006*b*) and higher in the breast of parakeets can be attributed to the flight ability of the species. Parakeets are strong flyers, using rapid wing beats, and have the capability of traveling up

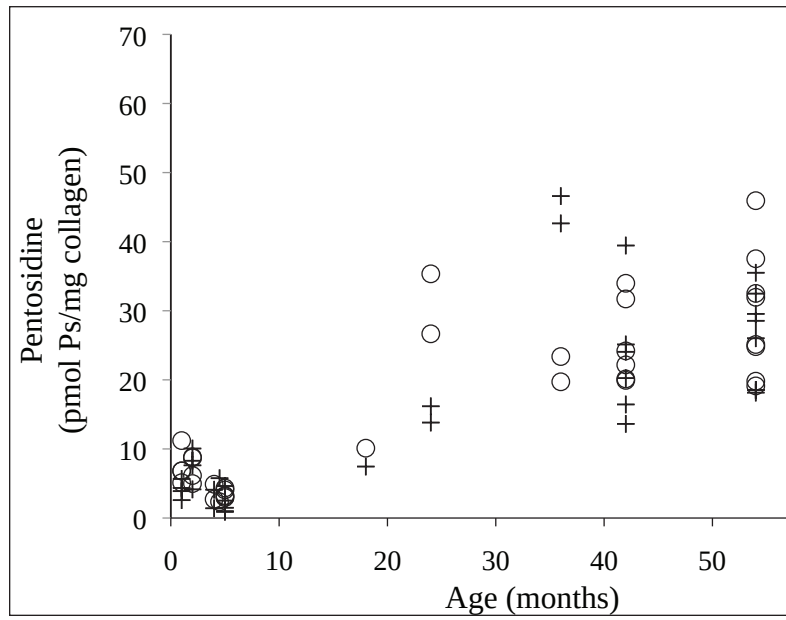

Figure 1. Distribution of pentosidine concentrations (pmol Ps/mg collagen) for known- and minimally-aged monk parakeets from Miami-Dade County, Florida, USA, in 2007. Known aged birds were from 1 to 18 months old, while minimally aged birds were 24+ to 60+ months old.

to 64 km to find food (Davis 1974), whereas ruffed grouse, are mainly land-based birds and seldom ever fly, except for short distances up to 0.4 km (Palmer 1962). We speculate that more oxidative stress for parakeets occurs in the flight muscles located on the breast (i.e., pectoralis major and pectoralis minor; Proctor and Lynch 2003) that elevate and depress the wing. This argument can be expanded to include vultures, which frequently soar and glide (Parrott 1970), thereby mainly using the deltoideus and extensor carpi obliquus muscles of the wing to rotate the wing while gliding (Proctor and Lynch 2003). Because vultures glide and soar, we speculate that oxidative stress may occur more in their wing muscles. Oxidative stress increases in muscles that go through excessive exercise (Ji 1999). Advanced glycation end-products, such as pentosidine, are highly associated with oxidative stress in birds (Iqbal et al. 1997, 1999; Klandorf et al. 1999). A future study could examine breast skin of migratory and nonmigratory birds of the same species to determine if strenuous use of flight muscles results in a higher pentosidine reading.

We speculate that there also may be differences in vascularity of the body regions of vultures and parakeets. Fast oxidative glycolytic and fast glycolytic fibers have a high density and a shorter distance to capillaries than other fibers that make up muscles (Butler 1991). These fibers are known to vary in proportion in different muscles and in different regions of the same muscles (Butler 1991), suggesting that vascularization can vary in a specific body part. If a region contains more capillaries, it will become perfused with blood, which may generate more oxidative stress and tissue cross-linking, resulting in higher pentosidine concentrations (Madamanchi et al. 2005). Amount of vascularity and oxidative stress may also explain why there were variations in pentosidine concentration between the different skin sizes. A future study comparing pentosidine from multiple skin samples of varying vascularity from regions of the patagium (and then breast) of the same subject could test if oxidative stress brought on by different quantities of vascularization influences pentosidine.

Statistically, the results are favorable for producing age estimates using 4- to 20-mm-diameter skin biopsies. In handling the tissue, we noted difficulty in obtaining and maneuvering the 4-mm-diameter skin samples compared to the larger sizes. The variation between the samples is of concern for producing an accurate age estimate for an individual bird. Due to this variability in pentosidine, we recommend that age curves be developed using only 1 specific skin-size. To make this procedure as minimally invasive as possible for live birds, we recommend that in future studies a smaller skin size be taken. Six-millimeter-diameter biopsy punches have been used in dermatology studies of live birds (Nett et al. 2003). For ease of handling and processing, we recommend using a 6-mm-diameter biopsy punch for pentosidine tissue procurement when determining the age of live birds.

The data from these studies suggest that either location can be used to produce an age estimate. Pentosidine variability between the samples is a concern here, as well. Therefore, we recommend that only 1 location be used for all future development of age curves and for sample collection. If pentosidine-based aging through pentosidine reading of birds becomes a wildlife tool, then the patagium seems like the least-invasive location to collect biopsies.

Both the cormorant and wild-bird age curve produced similar age estimates, suggesting that either of these curves will adequately estimate age for vultures until a vulture-specific age curve is developed. When comparing general age estimates based on physical characteristics to those determined from the age curves, we found good correspondence for most of the vultures. However, the validity of these estimates cannot be determined, as none of the vultures was of true known age. Further research involving known-aged vultures is needed to confirm the age estimations produced from this study.

The differences in parakeet age estimations can be attributed to the differences in pentosidine between skin locations. When comparing the estimated ages to the known-ages of the parakeets, most age estimates were accurate to within 6 months. This suggests that both estimated ages could still be considered extremely accurate (i.e., within 6 months), but, for consistency, we recommend that only 1 location, the patagium, be used in future aging studies.

This study also confirmed previous research showing pentosidine accumulation with age in birds (Iqbal et al. 1999; Chaney et al. 2003; Fallon et al. 2006*a*,*b*). A positive linear correlation in pentosidine accumulation with actual and minimal age was apparent for parakeets. A positive linear correlation appears to be present with vultures, as well, but until the vulture ages can be validated, we cannot confirm this. Species, genera, or family-specific age curves will need to be developed to age birds accurately. Until then, we are confident that use of pentosidine-based age method on birds without actual age records can be useful in determining the appropriate age class and in determing the hierarchy of age for individuals of a population.

To advance the usefulness of this aging technique, the next step will be to obtain pentosidine measurements from living birds. Consideration for their health and welfare must be of utmost importance. We recommend that 6-mm diameter skin samples be collected from the patagium of suitably sized living birds. Birds that often are fitted with patagial tags (e.g., vultures; Wallace et al. 1980), other raptors, and ravens (*Corvus* spp; Kochert et al. 1983) can be sampled in this manner. This technique may be feasible for use with smaller birds, as muscle biopsy samples have been successfully gathered from white-throated sparrows (*Zonotrichia albicollis*; Westneat 1986) and European starlings (*Sturnus vulgaris*; Romagnano et al. 1989) in the field with no deleterious effects. A captive study could be done to test the feasibility of taking 6-mm-diameter skin samples from the patagium of smaller birds, but at this time this technique is not recommended, as it is likely to be too invasive. When this technique is used in the wild, we anticipate that all sampling can occur in the field without the need to bring any birds into captivity.

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