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Development and evaluation of a minimally invasive sampling technique to estimate the age of living birds

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Development and Evaluation of a Minimally Invasive Sampling Technique to Estimate the Age of Living Birds

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ABSTRACT

Development of a Minimally Invasive Sampling Technique to Estimate the Age of Living Birds

Crissa K. Cooey

Using pest species in initial studies of pentosidine (Ps) aging research for birds may be the catalyst to discovering more effective population control strategies for pest, invasive, and hard to manage birds. Pentosidine is an irreversible, stable, fluorescent, collagen cross-link, created through the Maillard reaction, which has been found to accumulate throughout the lifetime of an organism in various body parts such as skin, lens crystalline, and dura matter. Pentosidine assays are more accurate at determining the age of adult birds in comparison to plumage coloration, eye and mouth color, feather wear, and molt sequences due to the discovery that Ps accumulates with age in the skin of birds. Past studies, however, have only taken place on deceased birds. To be considered a more generally useful tool for wildlife management studies, a procedure to obtain skin samples from living birds is needed. The objective of this project was to develop a minimally invasive sampling technique to age live birds through Ps analysis by 1) determining if differences exist in Ps concentration between the breast and patagium of black vultures (*Coragyps atratus*), monk parakeets (*Myiopsitta monachus*), and double-crested cormorants (*Phalacrocorax auritus*), 2) determining if differences exist in Ps concentration of 6 mm^2 and 20 mm^2 skin samples, and 3) determining if healing rates differ between the breast and patagium and between wounds closed with tissue glue and wounds closed with sutures. Pentosidine concentrations were similar between the breast ($\bar{x}$ = 8.9 pmol/mg collagen, SE = 0.55) and patagium ($\bar{x}$ = 8.9 pmol/mg collagen, SE = 0.51) of black vultures (P = 0.97) as well as the breast ($\bar{x}$ = 11.2 pmol/mg collagen, SE = 1.10) and patagium ($\bar{x}$ = 10.6 pmol/mg collagen, SE = 1.10) of deceased double-crested cormorants (P = 0.10). Pentosidine, however, was significantly higher in the breast ($\bar{x}$ = 15.9 pmol/mg collagen, SE = 1.30) than the patagium ($\bar{x}$ = 11.5 pmol/mg collagen, SE = 1.10) of monk parakeets ($P < 0.0001$). The Ps concentration was marginally higher in 6 mm^2 skin samples ($\bar{x}$ = 12.6 pmol/mg collagen, SE = 1.19) when compared to 20 mm^2 skin samples ($\bar{x}$ = 11.3 pmol/mg collagen, SE = 1.23) of cormorants (P = 0.02). Four new age curves were developed for cormorants (our linear breast skin age curve, our curvilinear breast skin age curve, our linear patagial skin age curve, and our curvilinear patagial skin age curve) and compared to the original Fallon age curve. Age estimates and actual ages for cormorant breast and patagial skins were found to be similar when using our linear and curvilinear breast and patagial skin age curves, but there were significant differences between actual and estimated ages for breast and patagial skin when using the Fallon age curve (P <0.0001 and P <0.0001 respectively). The mean estimated ages for all 5 age curves were found to be accurate to within approximately 1½ years (17.4 months). For 6 mm^2 skins, there was a marginal difference between real and estimated age when using our curvilinear patagial curve (P = 0.04), but real and estimated ages were similar for 6 mm^2 skins using our linear patagial curve and 20 mm^2 skins using our linear and curvilinear patagial curve. The mean estimated ages for the 2 age curves were found to be accurate to within approximately 2½ years (28.3 months). Seven living cormorants were caught at Bluff Lake, part of Noxubee National Wildlife Refuge in Mississippi, and were used to test the live sampling protocol. After a 6 mm^2 biopsy was removed from the breast and patagium, the birds had their wounds closed with either tissue glue

(n = 3) or sutures (n = 4). Wounds closed with tissue glue ($\bar{x}$ = 14.5 days, SE = 1.12) healed significantly faster than those closed with sutures ($\bar{x}$ = 17.3 days, SE = 0.66) (P = 0.0003) but the healing rate was similar for the breast ($\bar{x}$ = 15.9 days, SE = 1.36) and patagium ($\bar{x}$ = 15.8 days, SE = 1.85) (P = 0.79). Our finding is that live sampling can be safely done for live birds. Our recommendations are to live sample birds from the patagium with a 6 mm^2 biopsy punch and to close the wounds with tissue glue. Use of this technique could provide insight into senescence, reproductive success, and behavioral changes for different adult age classes as well as improve management strategies for pest and endangered/threatened species.

This thesis is dedicated to
all of my former biology teachers
and the staff of the Good Zoo at Oglebay in Wheeling, West Virginia.
My experience with all of them was an inspiration
to dedicate my career toward wildlife.

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CHAPTER I

Literature Review of Pentosidine Analysis as a Method of Estimating the Age of Birds

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RH: Cooey • Literature Review of a Bird Aging Technique

Literature Review of Pentosidine Analysis as a Method of Estimating the Age of Birds

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ABSTRACT Using pest species in pilot studies of pentosidine aging research for birds may be the catalyst to discovering more accurate conservation strategies for endangered and threatened birds. Three species of pest birds (black vultures [*Coragyps atratus*], double-crested cormorants [*Phalacrocorax auritus*], and monk parakeets [*Myiopsitta monachus*]) will be used to refine pentosidine assay techniques, as well as be used in a live sampling study. Black vultures are considered a pest because of their numerous collisions with aircrafts and livestock depredation. Double-crested cormorants are pests mainly due to the damage they cause at aquaculture ponds in the southeast U.S. The introduced monk parakeet will cause some damage to crops, but the nests they build are a nuisance and hazard to electrical companies. Pentosidine assays are more accurate at determining the age of adult birds in comparison to plumage coloration, eye and mouth color, feather wear, and molt sequences and it takes a shorter amount of time to complete than banding studies. Pentosidine is an advanced glycation end product created through the Maillard reaction. It is an irreversible, stable, fluorescent, collagen cross-link that has been found to accumulate throughout the lifetime of an organism in various body parts such as skin, lens crystalline, and dura matter. Past studies have discovered that pentosidine accumulates with age in the skin of birds, which makes it a promising biomarker that can be used to estimate the age of birds. A minimally invasive sampling method will need to be created before live sampling birds for pentosidine assays takes place. Considerations include the size of the skin

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sample taken, the location of the biopsy site, and the closure method for the wound. If pentosidine assays prove to be successful in aging living birds, this procedure could be valuable for research on migration, senescence, cooperative-breeding, life-table demographics, pest management, and conservation of birds. The following studies will investigate the optimal location to take a skin sample and the minimal size of skin that can be taken. Live sampling one of the pest species will also take place to determine if this is a feasible procedure to be used on living birds, as well as determine which closure method works the best for treatment of the wounds.

KEY WORDS advanced glycation end products (AGEs), avian aging, breast skin, *Coragyps atratus*, Maillard reaction, *Myiopsitta monachus*, patagium, pentosidine, pest species, *Phalacrocorax auritus*, skin samples

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Determination of age in individual birds can provide important information with respect to their biology and behavior. For example, zoologists working in captive breeding programs have been trying to identify factors in individuals important to enhancing the success of breeding programs. The oriental crested ibis (*Nipponia nippon*) captive breeding program was proven more successful when matched pairs had similar ages (Fulai et al. 1995). Fulai et al. (1995) commented that the youngest matched pair was unsuccessful, because they may not have been at the correct breeding age.

Pest species may be the catalyst for developing aging models and refining methods including aging endangered or threatened species in Species Survival Programs (SSPs). Certain methods of sampling for pentosidine (Ps) assay require destructive sampling of study subjects. Because some pest species are abundant and managed by lethal means, skin samples are

relatively easy to obtain. Samples from these pest species provide an opportunity to compare skin sampling methods and to refine and evaluate the potential for non-lethal sampling. If smaller non-lethal skin samples can be successfully obtained, then studies can be done in the future to determine the age structure of threatened and endangered species without being concerned that the sampling method will kill their study species.

Once a pest species exceeds societal acceptance capacity, management may be initiated to control or reduce damages or other nuisance activities. This wildlife damage management sometimes incorporates lethal control measures. Age estimates prove useful in developing life tables, pre-management modeling and to determine how many birds need to be euthanized to maintain population levels within social acceptance capacities. Bird banding studies often take a long time to acquire useable age structure data. Aging a random sample of birds by analyzing pentosidine in their skin will give information on the age structure of the population quickly (within a matter of months instead of years).

For the purpose of this study, we are using 3 pest species (black vultures [*Coragyps atratus*], double-crested cormorants [*Phalacrocorax auritus*], and monk parakeets [*Myiopsitta monachus*]) to determine if pentosidine assays are a viable aging method for living birds. If it is, this method 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 management and conservation strategies.

STUDY SPECIES

Black Vultures

General Description.- Black vultures are New World vultures and are part of the Cathartidae family, opposed to the Old World vultures, which are part of the Accipitridae family (Proctor

and Lynch 1993). They are found in the southeastern United States, ranging from Pennsylvania to Texas, with a separate range from southern Arizona into South America (Cornell Lab of Ornithology [CLO] 2007, U.S. Geological Survey [USGS] 2007*a*). They are approximately 56 cm long with a 137 cm wingspan (CLO 2007, USGS 2007*a*). Black vultures are entirely black, with the exception of white outer primary feathers and a gray unfeathered head, neck, and legs (CLO 2007, USGS 2007*a*). Both the juveniles and the adults have similar plumage (CLO 2007, USGS 2007*a*). They also have a short, squared off tail and a dark, hooked beak (CLO 2007, USGS 2007*a*). While soaring, typically in flocks (CLO 2007), they hold their wings flat (USGS 2007*a*). They will flap their wings more frequently in flight before soaring again, compared to turkey vultures (*Cathartes aura*) (Avery and Cummings 2004, CLO 2007, USGS 2007*a*). The black vulture's call is described as hisses, grunts, or barks (CLO 2007, National Wildlife Federation [NWF] 2007). Instead of retreating to individual nests, they form large communal roosts at night, throughout the year (Rabenold 1986). Black vultures have been reported to live as long as 25 years (USGS 2007*b*).

Reason for Study.- Breeding bird surveys (BBS) have indicated that black vultures have been steadily increasing at a rate of 2.99% yearly since 1967, but rates rose to 4.97% annually from 1990 to 2002 (or 5.97% annually since 1990 according to the Christmas Bird Count [CBC]) (Avery 2004). Black vultures are considered pest species 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). Black vultures like to roost on utility poles, power station transmitters, and private residences, making them undesirable “neighbors” (Fitzwater 1988). Vultures favor airports because of abundant road kill on the tar mats and on the roads leading to the facility and because they like to

soar on thermals created by warm air rising from the runways at airports (Satheesan and Satheesan 2000). In Fort Myers, FL, vultures perch on Low Level Wind-shear Alert Systems (LLWAS), often damaging the structures (Avery and Genchi 2004). Black vultures create a live barrier while soaring in flocks; this combined with their body mass and poor reflexes make black vultures extremely dangerous to aircrafts (Satheesan and Satheesan 2000). Black and turkey vultures are considered the top threat to civil and military aircraft collisions in the U.S. (Dolbeer et al. 2000). Between 1955 and 1999, 33 aircraft crashes (27 military and 6 civil) were caused by collisions with 7 different species of vultures throughout the world (Satheesan and Satheesan 2000). In 11 of these crashes, 21 lives were lost (Satheesan and Satheesan 2000). In the U.S black vulture collisions with aircrafts result in $10-17 million in damage per incident (Satheesan and Satheesan 2000). Although most collisions occur in the airfield environment (e.g., fighter jet crashes occurred most often during low level cruise [Satheesan and Satheesan 2000]), many collisions have been found to cause substantial damage during mid-flight (Dolbeer et al. 2000). This indicates that vulture populations need to be controlled not only at the airport, but also in a designated radius around the airport (e.g., removing food sources in a 200 km radius around a military airfield reduced populations of vultures in India) (Satheesan and Satheesan 2000).

Vultures not only feed on carrion, but they will attack, kill, and eat domestic animals (Avery and Cummings 2004). Eighteen states reported black vulture depredations from 1997 to 2002 (Avery and Cummings 2004). Over half of the kills involved cattle, with many kills involving young and newborn calfs (Avery and Cummings 2004). Kills typically involve 20-60 black vultures (Avery and Cummings 2004). Methods to keep vultures away from cattle include harassment by firing a .22 caliber rifle, removing dead livestock and road-killed animals from the

area, roost dispersal with effigies, trapping and relocation, and lethal control (Avery and Cummings 2004).

Double-crested Cormorants

General Description.- Double-crested cormorants (hereafter cormorants) range from Alaska, Manitoba, and Newfoundland south to Mexico and the Bahamas (CLO 2007, NWF 2007, USGS 2007*a*). They are approximately 69 cm long, with a wingspan of 127 cm (CLO 2007, USGS 2007*a*). They are a large bodied waterbird, with a long, thin neck, long, hooked bill, and long tail (USGS 2007*a*). Adults are entirely black, with the exception of an orange throat patch, and 2 black to partially or mostly white tufts of feathers (or crests) behind their eyes during the breeding season (CLO 2007, NWF 2007, USGS 2007*a*). The plumage can appear to have a green tinge to it in certain lighting (CLO 2007). Juveniles have brown plumage with a buffy breast, upper abdomen, and neck (CLO 2007, NWF 2007). Cormorants are often seen perching with their wings spread, a behavior where they are trying to dry themselves (CLO 2007, USGS 2007*a*). While in flight, double-crested cormorants have a slight crook in their neck, which distinguishes them from the other species of cormorant (CLO 2007, NWF 2007). Their call is described as a deep guttural grunt (CLO 2007, NWF 2007, USGS 2007*a*). Cormorants are known to live as long as 22 years (USGS 2007*b*).

They nest in colonies in trees or on cliffs near lakes, rivers, swamps, or coasts (CLO 2007, NWF 2007), or on the ground of isolated islands (CLO 2007). Cormorants make their nests out of sticks, seaweed, and man-made materials such as rope, fishnets, and plastic debris (CLO 2007, NWF 2007).

Reason for Study.- Cormorants were once a species of concern when environmental contaminants reached levels affecting cormorant populations regionally (Ludwig et al. 1989).

Consequently cormorants were placed under the protection of the Migratory Bird Treaty Act in 1972 (Glahn et al. 2000*b*). Regulatory protection from persecution, reduction in the use of pesticides (Tyson et al. 1999), and increased food availability, due to anthropogenic changes in the Great Lakes and man-made reservoirs, likely helped with a resurgence in cormorant numbers (Weseloh et al. 1995). The cormorant nesting population in the Great Lakes increased from 89 nesting pairs in 1970 to about 93,000 pairs in 1997 (Tyson et al. 1999). During this time, cormorants started inhabiting areas south of their original range, eventually leading to their colonization of Mississippi, Louisiana, Arkansas, and other southern states (Post and Seals 1991).

Sport fishermen in the Great Lakes are competing with cormorants for fish (Glahn et al. 2000*b*), because cormorants feed primarily on fish (CLO 2007). Wildlife managers are concerned with waterbird/cormorant competition over limited space and resources (Glahn et al. 2000*b*). Along with increased aquaculture cultivation of channel catfish (*Ictalurus punctatus*), crawfish, and baitfish, cormorant numbers have increased significantly over the past 25 years (Glahn and Stickley 1995). In 1998 there was an estimated 60,000 cormorants wintering in Mississippi (Werner and Hanisch 2003). Glahn et al. (2000*a*) found that their numbers have increased by about 250% in the Mississippi Delta region over this past decade. The production ponds for cultured fish in this area are highly susceptible to predation, especially by cormorants (Wywialowski 1999), with more foraging taking place at high density fish ponds (Werner and Dorr 2006). Increased winter survival of juveniles from readily available catfish and higher fat reserves of spring migrating adults may have contributed to cormorant population growth (Glahn and Brugger 1995). Glahn et al. (2002) estimated that cormorants are causing up to $25 million in damage annually from feeding at catfish aquaculture farms in the southeast United States.

Management practices, such as harassment, has proven difficult mainly because the result is only a temporary reduction in cormorant numbers (Mott and Boyd 1995). The U.S. Fish and Wildlife Service (USFWS) expanded the 1998 Public Resource Depredation Order (50 CFR 21.47) to allow removal of individuals from roosts near the aquaculture facilities (USFWS 2003). The only management method that has shown positive results so far is a combination of techniques, such as nest removal and harassment (Taylor and Dorr 2003).

Monk Parakeets

General Description.- Monk parakeets (also known as Quaker, cliff and gray-breasted parakeets) are a species of parrot that originated from central Bolivia, central Argentina and southern Brazil (Campbell 2000, Avery et al. 2002). They are approximately 28 cm long with a 48 cm wingspan, and weigh about 100 grams (Campbell 2000, NWF 2007). Females tend to be 10-20% smaller than males (Campbell 2000). They have bright green upperparts, their forehead and breast are gray, and the rest of the under parts are light green or yellow (Campbell 2000, NWF 2007). Their flight feathers are dark blue, and their tail is long and tapering (Campbell 2007, NWF 2007). They also have an orange bill (NWF 2007). Their call is a loud and throaty *graaa* or *skveet* (Campbell 2000, NWF 2007). Monk parakeets can live upwards of 6 years in the wild and 12-15 years in captivity (Pruett-Jones et al. 2007).

The monk parakeet is the only parrot that builds a stick nest, in a tree or on a man-made structure, rather than using a hole in a tree (Campbell 2000, NWF 2007). This species often breeds colonially, building a single large nest with separate entrances for each pair (Campbell 2000, Avery et al. 2002, Tillman et al. 2004, NWF 2007). In the wild, the nests can become quite large (i.e., 1- 4 m in diameter) (Campbell 2000, Avery et al. 2002, Tillman et al. 2004).

Reason for Study.- This species of bird has been used in the pet trade industry for at least the past 50 years. Because of that they have been introduced into the United States, beginning in the 1960s, from accidental or purposeful releases from former owners and pet store workers (Avery et al. 2002). In 1969, monk parakeets were reported as breeding in Miami, Florida (Stevenson and Anderson 1994). From 1971 to 1995, Christmas Bird Count (CBC) surveys documented 1,816 individuals from 76 populations in 15 states (van Bael and Pruett-Jones 1996). Population dynamics for this species fit an exponential model of population growth after analyzing the data (van Bael and Pruett-Jones 1996).

Their increasing numbers alone isn't the only problem in relation to human and animal interactions. They have the potential to spread Newcastle disease, but no literature has been published on this occurring in the U.S. (Fitzwater 1988). In their native South America, they are heavily regarded as agricultural pests, feeding on a variety of foods, including fruits (Mott 1973). They cause little damage, however, to agriculture in the United States (Avery and Tillman 2005). Their nest locations have given them a reputation for being a pest species in the U.S. They are especially fond of building nests on utility poles, transmission line support towers, and electric substations (Avery et al. 2002, Tillman et al. 2004). The sheer size of the nest and the materials it's made from can take its toll on the function of the transformers (i.e., the nest can result in arcing of current which causes damage to structures) (Avery et al. 2002). Damage can also occur when the nest materials get wet and inadvertently cause a short circuit, which results in a power outage (Avery et al. 2002, Avery and Tillman 2005). In 2001, monk parakeets created damages for electrical companies that totaled $585,000 (A. Hodges and C. Newman, unpublished data). Direct economic damages brought on by monk parakeet nests includes: 1) lost profit during outages, 2) costs of repairing equipment and restoring power, 3) costs for

mitigating monk parakeet populations, 4) indirect costs for personnel attending to the problem, and 5) costs to customers for loss of electricity (Avery et al. 2002).

Several remedies have been used to prevent monk parakeets from nesting on these man-made electrical structures that have met some success: 1) nest and bird removal, 2) owl effigies, and 3) laser beams (Avery et al. 2002). The introduction of an endemic protozoan parasite (i.e., *Sarcocystis falcatula*) may be a possible mitigation tool (Avery et al. 2002). This protozoan is lethal to psittacines but is not harmful to native birds of Florida (Avery et al. 2002). Another promising tool is feeding the parakeets the contraceptive diazacon (G. D. Searle Co., Omaha, Nebraska) (Yoder et al. 2007, Avery et al. 2008). Diazacon laced food has been effective in reducing reproductive success of monk parakeets both in the laboratory (Yoder et al. 2007) and in the wild (Avery et al. 2008). Most likely, some form of lethal removal will be necessary to control monk parakeet populations (van Bael and Pruett-Jones 1996).

COMPARISON OF AVIAN AGING METHODS

Researchers have developed several methods of aging birds, but they have proved to not be accurate past a certain age. Heinrich and Marzluff (1992) studied the change in mouth and tongue color to age common ravens (*Corvus corax*) from hatchlings to adults (3+ years). Changes in iris color in the Cooper's hawk (*Accipiter cooperii*) from yellow to orange to red have been used to age them as a juvenile or adult (Fagan 2008). "Skulling" or looking at the development and fusion of the bones in the skull is a way to age passerine birds up to about 6 months of age (Proctor and Lynch 1993). Plumage coloration and molt patterns also help identify a bird's age. For example, it takes approximately 5 years for the bald eagle (*Haliaeetus leucocephalus*) to develop a full white head (Dixon 1909). Jackson et al. (1992) studied age specific plumage characteristics and annual molt schedules in hermit (*Dendroica occidentalis)*

and Townsend's warblers (*Dendroica townsendi*). They observed that immature Townsend's warblers had well defined shaft streaks on their throat while the adults had faint or no shaft streaks (Jackson et al. 1992). If a certain species of bird will have 2 adult plumages in its lifetime, you can age these birds up to 3 years of age by looking at their plumage patterns (Amadon 1966, Jackson et al. 1992, Proctor and Lynch 1993, Thompson and Leu 1994). For birds with 2 alternating adult plumages, a breeding and non-breeding, the only way to transition from one plumage to another is by molting (Amadon 1966, Thompson and Leu 1994). Johnson (1963) found that molt sequences and schedules are rarely the same in any 2 species, and may be different for northern and southern birds of the same species. Along with comparing plumage coloration and molting patterns, some investigators look at feather wear to age birds (Saether et al. 1994). The primary feathers are examined for nicks or missing tips and barbule and hook wear (i.e., ability to reconnect the barbs) (Saether et al. 1994). This has been a reliable method of aging birds as either young or old (Saether et al. 1994). A disadvantage of the aforementioned methods is they are only reliable between 1 year (in shorter lived birds) and about 5 years of age (in longer lived birds).

Bird banding records can provide accurate ages for some, if not most, birds (Pollock and Raveling 1982, Sheaffer and Malecki 1995). At the time of banding, the correct age of the bird needs to be recorded for accurate age estimates when recaptured seasons later (Pollock and Raveling 1982, Sheaffer and Malecki 1995, USGS 2003). This is easiest to do when the birds are still juveniles (Nisbet 2001), but as mentioned above, aging is difficult to do for adult birds by looking at external features. Obtaining accurate ages from banding records is time consuming (years of banding and recapturing), expensive (cost of supplies, equipment, salaries) (Pollock and Raveling 1982, Sheaffer and Malecki 1995), cannot always be done (few recaptures or band

loss) (Coluccy et al. 2002), may not always be accurate (misidentifying correct age at banding) (USGS 2003), and can only take place on living birds (Pollock and Raveling 1982, Sheaffer and Malecki 1995) but other data such as site fidelity, migration patterns, etc. also can be obtained simultaneously (Pollock and Raveling 1982).

Alternative methods of aging birds have been studied over the past several decades. Focus has shifted from external examination to internal examination. Biomarker research appears to be promising for aging birds. Biomarkers, which show indications of biological age, correlate with chronological age (Ingram et al. 2001). One study involved looking at the shortening of telomeres as an indication of age in birds (Haussmann and Vleck 2002, Haussmann et al. 2003, Juola et al. 2006). Telomere restriction fragments (TRFs) have been documented to shorten with age in Adélie penguins (*Pygoscelis adeliae*), common terns (*Sterna hirundo*), tree swallows (*Tachycineta bicolor*), zebra finches (*Taeniopygia guttata*) (Haussmann and Vleck 2002, Haussmann et al. 2003) and great frigatebirds (*Fregata minor*) (Juola et al. 2006) but lengthen with age in the Leach's storm-petrel (*Oceanodroma leucorhoa*) (Haussmann and Vleck 2002, Haussmann et al. 2003). Unfortunately this method is not always reliable as no linear relation was found for TRF lengthening or shortening and time for the European shag (*Phalacrocorax aristotelis*) or wandering albatross (*Diomedea exulans*) (Hall et al. 2004).

Non-renewable structures, such as collagen, typically have the most noticeable age-related changes (Kohn and Schnider 1982). With age, there is a loss of elasticity in skin, arteries, lungs, and joints due to structural change in collagen (Kohn and Monnier 1987). Collagen in these tissues becomes less soluble, less digestible by collagenase, less expandable, and more resistant to heat denaturation with increasing age (Schnider and Kohn 1982). A more reliable

biomarker for bird aging may be a collagen crosslink. One collagen crosslink, known as pentosidine, is created by the Maillard reaction.

MAILLARD REACTION, ADVANCED GLYCATION END PRODUCTS (AGEs), AND PENTOSIDINE

Louis Camille Maillard (1912) discovered that by slowly heating 1 part glycine and 4 parts glucose together in water, the concoction turned a yellow-brown color after 10 minutes. This discovery led to the description of the Maillard, or nonenzymatic glycosylation, reaction. The Maillard reaction has been studied extensively to explain the occurrence of yellowing in long-lived proteins such as collagen and lens crystalline (Monnier and Cerami 1981). The Maillard reaction begins when a sugar aldehyde or ketone and a free amino group react through nonenzymatic condensation (Monnier 1989). A Schiff base with a free amine forms from this reaction, rearranging to create the Amadori product, a stable ketamine configuration (Monnier 1989, Sell et al. 1998). The Amadori product then undergoes a series of complex reactions, which leads to the formation of polymeric, yellowish, fluorescent, and crosslinking materials known as advanced glycation end products or AGEs (Sell et al. 1998). These products are irreversible chemical modifications of protein (Sell and Monnier 1989*b*), thermodynamically stable, and accumulate in long-lived biological molecules (Monnier 1989). At this point the Maillard reaction would be terminated *in vivo*, but if the Maillard reaction terminates *in vitro*, polymerization occurs, creating brown solutions, rich in melanoidins (Monnier 1989).

Pentosidine is a recently discovered fluorescent AGE, first isolated from human dura matter in 1989 (Sell and Monnier 1989*a*). It is formed from the reaction of a sugar (i.e., glucose, fructose, or ascorbate) with a protein (Grandhee and Monnier 1991). Glucose itself or Amadori adducts of glucose and protein also can be sources of pentosidine (Dyer et al. 1991).

Pentosidine's structure consists of a ribose molecule crosslinked with a lysine and arginine residue (Figure 1) (Sell and Monnier 1989*b*). Pentosidine has been found in a variety of tissues and organs, such as the lung, aorta, kidney glomeruli, trachea, heart muscle, dura matter, bone, lenses, human fibroblasts and glomerular mesangial cells (Monnier et al. 1993). Pentosidine also has been found to accumulate with age in the skin of a variety of mammals, such as humans, shrews, dogs, cows, pigs, and monkeys (Sell et al. 1996), and more recently birds (Iqbal et al. 1999, Chaney et al. 2003, Fallon et al. 2006*a,b*). Recent research has shown that pentosidine has accelerated the aging process in diabetic humans (Sell and Monnier 1990, Sell et al. 1992, Sell et al. 1998). Advanced glycation end products alter the structural properties of tissue proteins and reduce their susceptibility to catabolism (Sell and Monnier 1990). These changes contribute to the aging of tissues, which cause the development of diabetic complications through advancement by hyperglycemia (Dyer et al. 1993). Diabetic patients had Ps concentrations above the 95% CI for nondiabetic patients of the same age (Sell et al. 1992). All of this research suggests that Ps might be the key to the aging process (Iqbal et al. 1999) and has been proposed to serve as a senescence crosslink.

PENTOSIDINE ACCUMULATION IN AVIAN SKIN

Mammals have been reliably aged in the past through teeth wear, graying of hair, and wrinkling of skin (Finch 1976), and pentosidine concentration analysis only strengthened the age estimate. Iqbal et al. (1999) proposed that birds could be aged through pentosidine concentration analysis from skin samples. Iqbal et al. (1999) discovered that pentosidine accumulated in a linear fashion with age in euthenized broiler breeder chickens (*Gallus gallus domesticus*) skin, but noticed that Ps concentration in avian skin was 1,000 fold lower than in mammalian skin. This observation could explain why birds live a lot longer than mammals of

similar size. Birds have higher metabolic rates (2.5 times higher), higher body temperatures (3^{o} C higher), and higher plasma glucose (2-6 times higher) than mammals, which typically accelerates the aging process (Iqbal et al. 1999). Research by Chaney et al. (2003) found that pentosidine increased linearly with age in various species of wild birds (R^2=0.73), further supporting Iqbal's claims that pentosidine can be used as a biomarker for chronological age in birds.

Verzijl et al. (2000) calculated that the first reasonable estimates of half-lives of cartilage collagen and skin collagen were 117 years and 15 years respectively. This suggests that pentosidine concentrations, being an irreversible protein modification that accumulates with age, can be determined from collagen years after an individual has been deceased. Fallon et al. (2006*a*) were the first to investigate if Ps could be obtained from study skins. Pentosidine concentrations obtained shortly after death were not significantly different from pentosidine concentrations obtained 1 year after the study skin was prepared (Fallon et al. 2006*a*). Fallon et al. (2006*a*) also were the first to discover that pentosidine concentrations may vary in different body parts. They discovered that Ps had higher concentrations in the thigh and wing compared to the breast (Fallon et al. 2006*a*). Finally, Fallon et al. (2006*b*) discovered that pentosidine accumulates faster in shorter-lived birds and slower in longer-lived birds. This suggests that an age curve must be created for each species, or at least each taxonomic order of birds to give accurate age estimates. It would not be wise to use an age curve for a ruffed grouse (*Bonasa umbellus*) (life span up to 10 years in captivity [Fallon et al. 2006*a*]) to age macaws (*Anodorhynchus hyacinthinus*) (life span up to 75 years, but average 40-50 years [Mijal 2001]).

MINIMALLY INVASIVE SAMPLING

It is important for biologists to use a sampling technique that will be as minimally invasive as possible when sampling wildlife (Griffin and Gauthier 2004). Sometimes the slightest change to an individual can cause drastic effects to occur. The slightest damage to an appendage in males of certain species may result in poor or no reproductive success for that individual. For example, black grouse (*Tetrao tetrix*) will select mates based on tail length (Rintamāki et al. 1997). By damaging the symmetry of a particular individual, in this case damaging the tail feathers, it is possible to cause the most "fit" individual to not produce any offspring (Moller and Thornhill 1998). This may result in next year's age class having genetic phenotypes significantly different from their parent's age class phenotypic demographics. Birds have few sensory cells in their skin (Proctor and Lynch 1993), so taking a skin sample shouldn't cause a lot of pain. Nonetheless, it is important to keep discomfort to a minimum when taking samples from living organisms. Traumatized animals may show a change in their normal behavior, which can defeat the purpose of a particular study (Griffin and Gauthier 2004). Animals that experience pain when they are released may become weakened and less alert, making them an ideal target for most predators (Griffin and Gauthier 2004). This could be detrimental to a study if it involves endangered or threatened species.

A well thought out procedure will need to be developed before taking skin samples from living wild birds becomes a viable wildlife aging technique (Griffin and Gauthier 2004). A variety of biopsy instruments can be used to obtain skin samples. Karesh et al. (1987) investigated whether skin biopsies could be obtained from large mammals (e.g., gorillas [*Gorilla gorilla*]) in captivity, with no restraint of the animal, by shooting a biopsy instrument out of a dart pistol or rifle. Silverman et al. (2007) took skin biopsies in dogs using monopolar

electrosurgery, CO_2 laser, radiowave radiosurgery (RWRS), a scalpel, and biopsy punches. Of these instruments, skin charring did not occur on samples taken with the scalpel or biopsy punch (Silverman et al. 2007). For a study on birds, a biopsy punch may be the best instrument to use in sample collection. Biopsy punches come in a variety of sizes (1 mm – 8 mm diameter) and take consistently sized circular pieces of skin every time, requiring no additional measuring. Biopsy punches are readily available and are economically priced for field research. Using a biopsy punch to take a skin sample from wild birds will take only the required amount of skin needed for analysis. Samples can be taken quickly and accurately, causing the least amount of stress and discomfort on the birds (Nett et al. 2003). It is suggested that only a 6 mm^2 sample of skin is needed to determine an accurate age for any given bird (J. A. Fallon West Virginia University, unpublished data).

Fallon et al. (2006*a*) found that pentosidine levels can vary in different parts of a bird's body. Skin samples should be consistently taken from the same body part to eliminate variance in results. Fallon et al. (2006*a,b*) aged birds from skin samples taken from the breast of deceased birds. If this sampling becomes a standard practice for living birds, little harm must be done to major muscle groups on the bird. Damaging the pectoralis major muscle of the chest, which provides most of the force for a downward stroke in flight, could severely reduce flight capability (Proctor and Lynch 1993). The patagium on the wing of the bird may be a better place to sample living birds. There are fewer muscles in the wing and the muscles used in flight are located around the sternum (Proctor and Lynch 1993). There are four patagial areas on the wing: 1) the prepatagium (hereafter patagium), 2) metapatagium, 3) post patagium, and 4) alular patagium (Figure 2) (Lucas and Stettenheim 1972). The free edge of the patagium is directed anteriorly (Lucas and Stettenheim 1972). The mediocaudal boundary is the anterior margin of

the biceps brachii and the distocaudal boundary is the forward edge of the extensor metacarpi radialis muscles (Lucas and Stettenheim 1972). Some of the research will take place with skin from this region of the wing. The patagium has often been used as a location to place permanent ID tags. However, some problems have arisen from using these tags. The Carnaby's cockatoo (*Calyptorhynchus latirostris*) has become depredated more easily because the tags make them more visible to predators (Saunders 1988). Also, ruddy duck (*Oxyura jamaicensis*) males with patagial tags had fewer successful courtships than males without tags (Brua 1998). Few birds have died from being fitted with a patagial tag (Chapman and Chapman 1990). An American white pelican (*Pelecanus erythrorhynchos*) was found dead from emaciation when its bill got ensnared on the patagial tag (Chapman and Chapman 1990). However, some species of birds have had no problem with being fitted with a patagial tag. Smallwood and Natale (1998) state that the placement of patagial tags had no adverse effect on American kestrel (*Falco sparverius*) health. This suggests that the patagium may be an ideal location to take skin samples. Patagial tags pierce through both layers of skin of the patagium. A biopsy sample would only require one layer of the patagial skin to be removed. The patagium is made up of two layers of skin, some tendinous tissue, and the patagialis longus muscle (Proctor and Lynch 1993). The patagialis longus muscle can easily be seen under the skin, so samples can be taken from that area without causing harm to that muscle. There are fewer blood vessels in the wing (Proctor and Lynch 1993). If the feathers are separated enough and the veins are clearly seen in the patagium, then a skin sample can be taken without causing much bleeding. As with any type of surgery, morbidity and mortality can occur (Beal et al. 2000). By sampling around the veins of the patagium, there will be less occurrence of an infection reaching the blood stream, which will reduce the chance the bird will die (Beal et al. 2000, Muza et al. 2000).

Inflammation and infection are always risks when taking biopsy samples from any organism. Calrson and Allen (1969) noticed chickens had acute inflammatory reactions occur on their wounds. Typical signs of this response are heat, swelling and redness (Carlson and Allen 1969). As the inflammation subsides, lymphocytic hyperplasia may develop (Burke et al. 2002). The major concern during the surgical procedure is developing perioperative hypothermia. Perioperative hypothermia is known to reduce resistance to bacterial infection (Beilin et al. 1998) and increase blood loss (Schmied et al. 1996). Two ways to avoid developing postoperative complications, such as infection, is to keep the surgical duration as short as possible and apply anesthesia before cutting into the skin (Brown et al. 1997). Making the wound site as clean as possible also will prevent infection. In a study of 1,255 dogs and cats, the infection rate was only 4.8% when the wounds were cleaned (Beal et al. 2000). This is close to the 5% human infection rates from clean wounds (Cruse and Foord 1980). If inflammation or infection develops, there are several topical medications available that have been used successfully in avian treatment. These include silver sulfadiazine cream, nitrofurazone, gentamicin sulfate ointment, enzymatic debridement agents, yeast cell derivatives, camphor spirits, tincture of benzoin, and softening agents (Burke et al. 2002). Medications that are poorly absorbed by wound tissues should be avoided if possible including bacitracin, neomycin, and polymixin (Burke et al. 2002).

The biopsy site should also be closed before releasing the animal. Surgical glue and dissolvable sutures (2-0 to 5-0) are two methods of treatment that have been used in fieldwork (Small et al. 2004, Schwagmeyer et al. 2005). Small et al. (2004) used surgical glue to close the wound made to place a subcutaneous radio transmitter in white-winged doves (*Zenaida asiatica*). They found no adverse changes in the wounded birds when compared to the control birds (Small

et al. 2004). Both surgical glue and dissolvable sutures were successfully used to close implant sites in house sparrows (*Passer domesticus*) (Schwagmeyer et al. 2005). Superficial wounds in avian skin will heal typically in 10-14 days (Burke et al. 2002). This information suggests that as long as the sampling sites are cleaned and anesthesia is applied beforehand, the procedure is done as quickly as possible, and the wounds are sealed afterwards, the researchers do not need to worry that many of their subjects will develop inflammation or infections from the wounds.

IMPORTANCE OF KNOWING AGES IN BIRD STUDIES

Age has been a topic of study with migratory birds for years. Migration is typically done more often by females and immature (first winter) birds, whereas males and adult birds (second winter and older) are more sedentary (Smith and Nilsson 1987). Age can be a factor when it comes to early or late arrival after migration. Moller and DeLope (1999) found that 1 year old and 5+ year old barn swallows (*Hirundo rustica*) arrived later from spring migration than the birds of ages 2-4 years. Jones et al. (2002) found that for most species of migratory bird they sampled, there was no significant difference in the rate of mass gain at a stopover site with age. If the ages of the adult birds were known in this study, Jones et al. (2002) may have discovered a significant difference in rate of mass gain. But for that study, they only grouped the birds as adults (AHY) or immature (HY) (Jones et al. 2002). Knowing the age structure of the populations in these studies could tell us whether age is the reason why there is a difference in body mass for migrants or if there are other factors that influence weight more.

Cooperative-breeding birds are of interest for many studies. They have delayed reproduction, which is a characteristic of many long-lived birds (Brown 1987). These birds will begin breeding at a variety of different ages (Brown 1987). Knowing the age at their first breeding and then calculating their reproductive success over the years could explain if taking

several years to help raise other family member's offspring is worth the sacrifice of not passing on their own genetic material. This could be of great importance for threatened or endangered cooperative breeders.

Studies on senescence in the wild is extremely difficult, especially for long-lived species (Nisbet 2001). Birds are great subjects to use when studying senescence, because they live much longer than mammals of comparative size. Senescence is the decrease in reproductive value and the increase in mortality rates with age (Holmes et al. 2001), thought to arise from the accumulation of deleterious mutation and/or the negative pleiotropic effects late in life of alleles with a beneficial effect during early life states (Hamilton 1966). Hamilton (1966) wondered if a mutation occurring at a specific age in a bird's life would have an influence in its evolutionary process, thus affecting the individual's senescence. Birds are known to exhibit gradual senescence with a definite life span (Holmes et al. 2001). A decline in reproductive success can be seen in barn swallows (Moller and DeLope 1999). Reproductive success increased from ages 1-3 and then decreased slowly from 3-4 years, 4-5 years and even more with 5+ year olds (Moller and DeLope 1999). However, reproductive senescence appears to have little influence on seabirds. The common tern (*Sterna hirundo*), for example, is known to live up to 26 years and has reported to breed as old as 21 years (Nisbet et al. 1999). Further research of seabirds may give some more insight to why birds naturally live longer than comparably sized mammals.

Life-table demographics can be used in several ways in the conservation field: assess population status; diagnose the causes of poor population performance; to prescribe management tactics; and to make prognoses of population viability (Caswell et al. 2003). Knowing age demographics of bird populations can provide researchers with information to help them prevent

native populations from dwindling and invasive species populations from growing at an exponential rate.

The main value of a life table lies in what it tells us about the population's strategy for survival (i.e., life tables help us to understand the dynamics of populations) (Deevey 1947). Age-specific estimates of survival and reproduction are needed for assessments of population trends and population modeling (Caughley 1977). Knowing the age classes of wild populations of birds is important when evaluating survival and recruitment rates (Moen et al. 1991). For example, the correct identification of sub-adults tells us the recruitment of young born the previous year (Moen et al. 1991). Harris and Shepherd (1965) studied known-aged black brants (*Branta bernicla orientalis*) to determine their minimal age to start breeding and assess their reproductive potential. They found that up to 1/3 of the females in the population begin to breed at age 2, but no firm evidence was found on when males first started breeding (Harris and Shepherd 1965).

Numbers will fluctuate over time for any given population. A general rule for organisms with well-defined generations is that recruitment or reproduction occurs only over a short period of time, while mortality occurs continuously (Podoler and Rogers 1975). Because of this, changes in population density are often defined in terms of changes in mortality during particular stages of the lifecycle (Podoler and Rogers 1975). Knowing more about mortality rates at different ages will give researchers more insight into population fluctuations.

Time-specific life tables are valuable to a manager of exploited populations because they show the existence of strong year classes or help identify weak age classes (Deevey 1947). Management practices for populations with high numbers focus on the exploitation or manipulation of a particular life-history stage (Nicoll et al. 2006). An example of this is

harvesting eggs from nests, which will make reduced birth rates for the species immediately apparent (Nicoll et al. 2006). Harvesting eggs from the age class that has the highest birth rate will reduce the population growth of the species. However, this may only be a temporary solution to population regulation. Life-history theory suggests that if a particular fitness trait, such as fecundity, is manipulated (i.e., egg harvesting) then other fitness traits may have benefits or costs applied to them (Nicoll et al. 2006). By adjusting the natural balance of a population, individual life histories and population dynamics may become altered (Nicoll et al. 2006).

Invasive species or pest control can be a hit or miss practice, sometimes resulting in total failure while other times producing the desired results (Frederiksen et al. 2001). When pest control ends up failing, blame can be placed on a lack of understanding of population dynamics for the species being controlled (Frederiksen et al. 2001). Having a better understanding of life-cycle parameters such as survival, reproduction, etc., can be used to help predict how populations will respond to different treatments of eradication (Tuljapurkar and Caswell 1997).

Frederiksen et al. (2001) compared different models created to predict the population changes of great cormorants (*Phalacrocorax carbo sinensis*). Previous attempts to model cormorant population reactions to various management strategies were done by Lebreton and Gerdeaux (1996) and Bregnballe et al. (1997), but the only conclusions that they reached were that the cormorant population growth rate is more sensitive to changes in adult survival than in fecundity, and hunting or culling may reduce the population numbers and cause faster stabilization of the population if density-dependence is assumed to occur naturally. Culling is a common eradication practice for pest species (Duncan 1978, Frederiksen et al. 2001). This involves removing a large portion of the population, typically by lethal means (Duncan 1978, Frederiksen et al. 2001). Culling can regulate or even eliminate target species if carried out in a

density-dependent manner (Frederiksen et al. 2001). For example, culling practices reduced the breeding density of herring gulls (*Larus argentatus*) from 11.1 pairs per 100 m^2 to 2.3 pairs per 100 m^2 on the Isle of May in Scotland (Duncan 1978). When pest species are regulated by density-dependence at levels high enough to cause management problems, culling may have less effect than expected because reductions in population size are compensated for by increases in 1 or more life-cycle parameters (Frederiksen et al. 2001).

GOALS AND OBJECTIVES

The overall goal of our research is to assess the feasibility of applying the pentosidine aging technique to living birds. In Chapter 2, "Pentosidine Concentration Comparisons of the Breast and Patagium of Birds," we address the following objectives: 1) compare the concentrations of Ps in the breast and patagium for each individual bird tested and 2) determine if there is a significant difference in estimated age between the locations. We hypothesize that there is no significant difference in Ps concentrations between the breast or patagium for each individual bird, and there is no significant difference in estimated age generated for the locations.

In Chapter 3, "An *In Vivo* Pentosidine Aging Technique Evaluation for Double-crested Cormorants," we address the following objectives: 1) compare pentosidine levels in the breast and patagium of known-age cormorants, 2) develop and evaluate accuracy of aging models of known-aged cormorant samples taken from the breast and patagium, 3) compare pentosidine levels between a 6 mm^2 sample and a 20 mm^2 sample of skin from cormorants, and 4) compare the healing rate of 6 mm biopsy samples from the breast and patagium of living cormorants by suture closure or application of tissue glue. We hypothesize that there is no significant difference in Ps concentrations between the breast and patagium for the cormorants, there is no

significant difference in Ps concentration between the two different skin sizes, and there is no significant difference in the healing rate of skin from the 2 wound closure methods.

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FIGURES

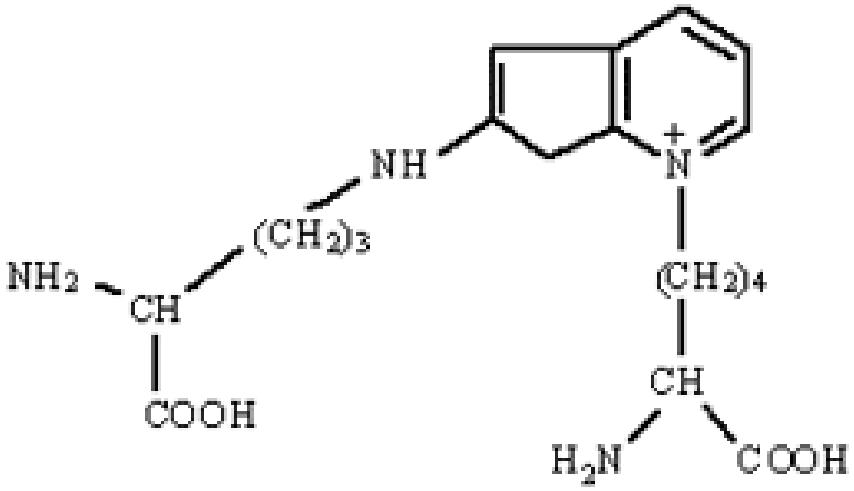

Figure 1. Molecular structure of pentosidine. It is comprised of a ribose compound connected to a lysine and arginine residue (Takahashi 2006).

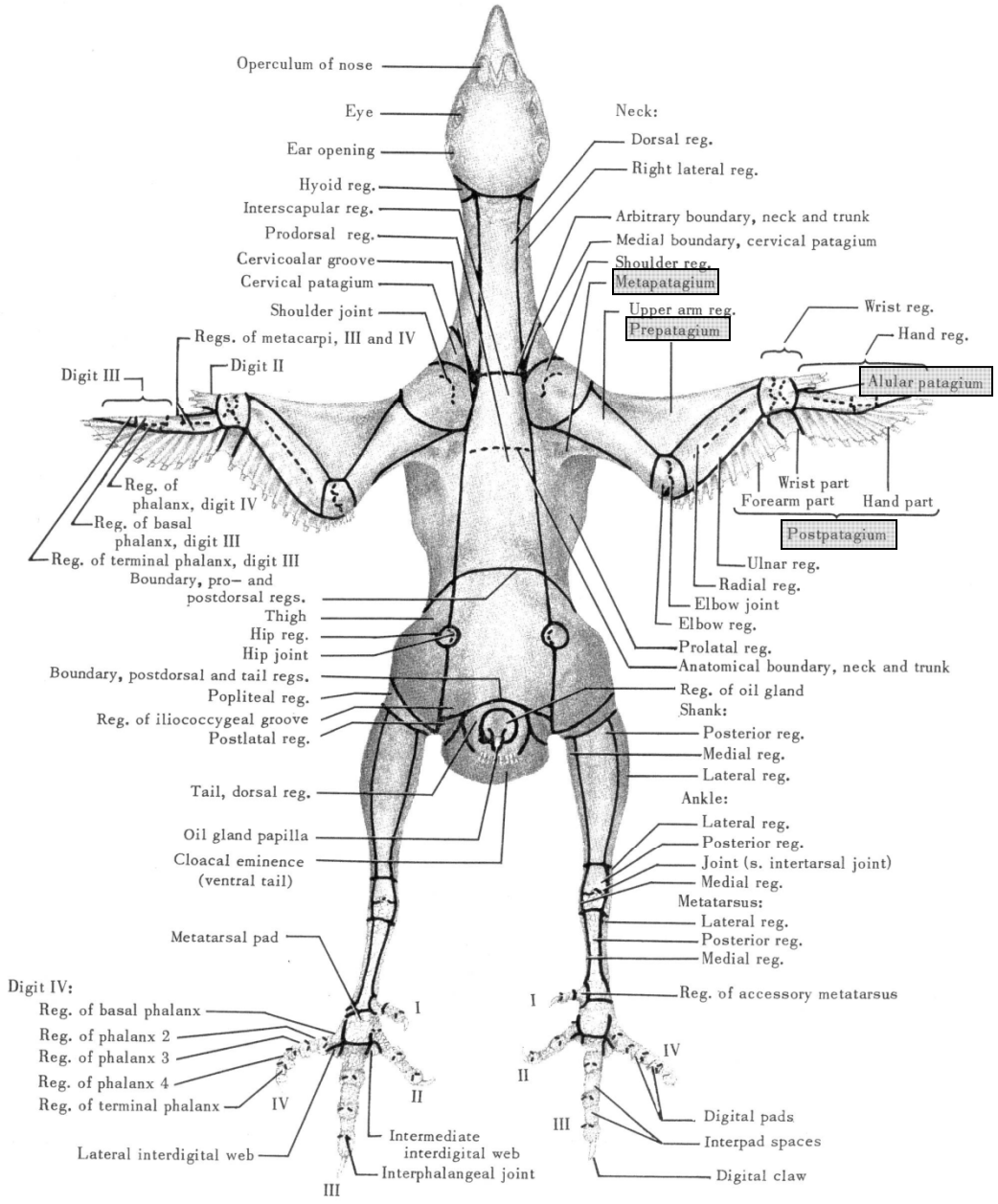

FIGURE 33.—Dorsal view of a male Common Coturnix showing regions. Abbreviations: reg(s)., region(s); s., synonym.

Figure 2. Anatomy of a bird's wing highlighting the 4 patagial areas of the wing: 1) prepatagium; 2) metapatagium; 3) post patagium; and 4) alular patagium (Lucas and Stettenheim 1972). The skin samples will be removed from the prepatagium.

CHAPTER II

Pentosidine Concentration Comparisons of the Breast and Patagium of Birds

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Pentosidine Concentration Comparisons of the Breast and Patagium of Birds

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ABSTRACT Accurate aging of birds is useful for evaluating reproductive success, life-table demographics, and senescence. Until recently, only banding studies have provided accurate ages for birds after they reach adulthood, although on occasion sample records are not completely accurate. Measurement with breast tissue pentosidine (Ps) from deceased birds can provide age estimates of unknown-aged birds. To be considered a more generally useful tool for wildlife management studies, a procedure to obtain skin samples from living birds is needed. For this reason, we investigated the patagium as a location to obtain a skin sample as an alternative to the breast. Our main objectives were to determine if Ps concentrations varied significantly between the breast and patagium of black vultures (*Coragyps atratus*) and monk parakeets (*Myiopsitta monachus*) and if age curves generated from breast skin can be used to estimate age for samples obtained from patagial skins. The Ps concentration for the breast ($\bar{x}$ = 8.9 pmol/mg collagen, SE = 0.55) and patagium ($\bar{x}$ = 8.9 pmol/mg collagen, SE = 0.51) of vultures were similar, but in

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This chapter is written in the style of *The Journal of Wildlife Management*

parakeets, Ps concentrations were higher in the breast ($\bar{x}$ = 15.9 pmol/mg collagen, SE = 1.30) than the patagium ($\bar{x}$ = 11.5 pmol/mg collagen, SE = 1.10). This indicates that a skin sample from either body part will reliably give the same estimated age for vultures but not for parakeets. Known and minimal ages for parakeets (n = 41) were compared to estimated ages, and for at least 88% of known-aged parakeets estimated ages were within 6 months of the actual ages when using the wild bird age curve generated from a previous study, suggesting that the Ps differences found between the breast and patagium in parakeets may not be biologically important. Overall our findings indicate that either sampling site can be used for Ps measurement to age living birds, but we recommend that the patagium be used to minimize stress to the bird.

KEY WORDS bird age, black vulture, breast skin, *Coragyps atratus,* monk parakeet, *Myiopsitta monachus*, patagium, pentosidine.

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Knowing the age of birds is valuable for studies on reproductive success (Brown 1987), increasing success in captive breeding facilities (Fulai et al. 1995), increasing accuracy of life-table demographics (Moen et al. 1991), and improving the understanding of senescence in birds (Hamilton 1966). Age specific traits, such as plumage colors or patterns (Amadon 1966, Jackson et al. 1992, Proctor and Lynch 1993, Thompson and Leu 1994), eye color (Dixon 1909), and mouth color (Heinrich and Marzluff 1992), can be different in juvenile and adult birds. However, estimating ages of sexually mature birds has proven difficult.

If the correct age of a bird is noted at the time of first capture (Pollock and Raveling 1982, Sheaffer and Malecki 1995), banding studies can provide a reliable age estimate (Sumner 1940, Pollock and Raveling 1982, Sheaffer and Malecki 1995). However, there are some limitations to banding studies. They are time consuming and expensive (Sumner 1940, Pollock

and Raveling 1982, Sheaffer and Malecki 1995), have numerous band losses and few recaptures (Coluccy et al. 2002), may not always be accurate (Moen et al. 1991), and can only take place on living birds (Sumner 1940, Pollock and Raveling 1982, Sheaffer and Malecki 1995). Age determination is limited to birds that have been previously banded.

Pentosidine (Ps) analysis can reliably age birds throughout their entire lifespan (Iqbal et al. 1999, Chaney et al. 2003, Fallon et al. 2006*a*,*b*). Pentosidine measurement also estimates age in a much shorter time span than it takes to complete a banding study. The Ps assay is a standardized objective procedure, but to date this tool has not been adapted to sample living birds, with the uncertainty of its use being a drawback.

Species-specific age curves developed from Ps analysis have been created for ruffed grouse (*Bonasa umbellus*), double-crested cormorants (*Phalacrocorax auritus*) (Fallon et al. 2006*a*), and bald eagles (*Haliaeetus leucocephalus*) (J. A. Fallon, Virginia Tech and C. K. Cooey, West Virginia University, unpublished data). Moreover, a general wild bird curve was developed from known-age birds of 29 different species (Chaney et al. 2003). To have a better understanding of how Ps varies for different orders, families, and genera of birds, more species-specific age curves need to be developed.

Most of the previous avian aging studies through Ps analysis have taken place with breast skin from deceased birds. To use Ps analysis as a wildlife tool for aging living wild birds, there is a need to create a minimally invasive sampling technique. Because the breast contains the major flight muscles for birds (Proctor and Lynch 1993), sampling skin from the breast could seriously impair flying ability. Marking birds with patagial tags has been a standard technique for many years (Marion and Shamis 1977, Wallace et al. 1980). Performed properly, insertion of a marker through the patagium has no deleterious effects on the health or flying ability of the bird

(Sweeney et al. 1985). The patagium also contains few veins (Proctor and Lynch 1993), decreasing the chance of bacteria entering the blood stream and causing an infection (Muza et al. 2000). The patagium, therefore, seems like a suitable location for obtaining skin samples from live birds.

A previous study by Fallon et al. (2006*b*) indicated that Ps concentrations in 6 ruffed grouse varied for different sampling sites and were higher in the patagium compared to the breast (Fallon et al. 2006*b*). The skin samples were approximately 2 cm^2, a size which would not be feasible to take from living birds, especially those that are small (e.g., most passerines). Before live sampling wild birds can be attempted, it is necessary to determine if age curves from breast skin can be used to estimate ages for patagial skins or if new curves need to be developed based on patagial skin. In addition it is also essential to determine the amount of skin that can be taken for analysis. Using the Fallon et al. (2006*b*) procedure, Ps concentrations of the breast and patagium were determined in monk parakeets (*Myiopsitta monachus*) and black vultures (*Coragyps atratus*).

Monk parakeets (also known as Quaker, cliff and gray-breasted parakeets, hereafter parakeets) are small omnivorous birds that were introduced to the United States via the pet trade from South America in the 1960s (Long 1981). Banding studies in their native range indicate a potential lifespan of at least 6 years, but age structures of invasive populations in the USA are unknown (Spreyer and Bucher 1998). Black vultures (hereafter vultures) are long-lived birds with a potential life span in excess of 20 years (Buckley 1999). Population age structures and key aspects of their life history such as age of first breeding remain unknown, because these birds cannot be aged reliably (Blackwell et al. 2007). Thus, for each of these species, development of a verifiable age estimation method is warranted. Based on the Fallon et al.

(2006*b*) original finding, we hypothesize that the concentration of Ps will be significantly higher in the patagium of the wing compared to the breast.

METHODS

In 2004, we collected 30 vultures as road kills (n = 1) or as part of a vulture population management program (n = 29; U.S. Department of Agriculture [USDA] Wildlife Services 2003). We collected approximately 150-mg skin samples from the breast of the vultures at necropsy, froze samples in distilled water, and overnighted them to West Virginia University (WVU), Morgantown, WV, USA in 2004 for analysis. We retained the carcasses at the USDA National Wildlife Research Center (NWRC) field station in Gainesville, FL, USA where they were frozen. 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, PA). We overnighted these skin samples to WVU for analysis. Based on the Fallon et al. (2006*b*) finding that pentosidine remained stable for at least 1 year in museum study skins, we assumed that pentosidine remained stable for at least 1 year while frozen.

In January 2007, we collected skin samples from parakeets at the USDA NWRC field station in Gainesville, FL. Known-age birds were those that had either been raised in captivity or had been collected in the field as juveniles. Others were collected or trapped as adults and therefore only a minimum age estimate was possible. We allowed the parakeets (n = 97) to thaw for 30 minutes – 1 hour before skin samples were collected. We removed skin samples (~ 150 mg) from the breast, as well as the entire patagium from the left wing, from each parakeet and placed them in labeled plastic bags, which were frozen until analysis.

The breast samples of the vultures were analyzed in a previous study from 2004 (J. A. Fallon, West Virginia University, unpublished data). We compared Ps concentrations from the

patagial skin samples to the initial Ps concentrations from the breast. In the previous study, Ps concentrations from 4 different sized samples (4 mm^2, 6 mm^2, 8 mm^2, and 20 mm^2) of vulture breast skin were compared. Initial findings indicated that there was no significant difference in Ps concentrations which suggests that a 6 mm^2 skin sample could be accurately aged from an age curve generated from the 20 mm^2 skin data. In view of this observation we collected 6 mm^2 skin samples from the patagium of the vultures and compared then to the 6 mm^2 breast skins previously analyzed to determine differences in Ps concentration between the breast and patagium.

For parakeets, we processed skin samples of approximately 40 mg (20 mm^2) to determine if differences exist between Ps concentrations in breast and patagial sampling sites. We prepared all skin samples as described by Iqbal et al. (1999).

Skin Preparation

We allowed skin samples from the freezer to thaw overnight in the refrigerator or for 30 minutes – 1 hour on the lab bench. We scraped the skins with a scalpel blade to remove any remaining feathers, feather shafts, epidermal layers, muscle, and adipose tissue, until the skins were almost transparent, and then minced. We placed approximately 40 mg of the minced skin into a properly labeled 12 x 100 mm capped glass tube. We placed the tubes in the freezer if delipidation was not going to occur immediately.

Delipidation

We performed delipidation to remove any remaining fat. We added a chloroform/methanol (C/M) solution (2 parts chloroform to 1 part methanol) (5 ml) to each tube and then we placed the samples on a 360° rotating agitator in a 4° C cold room for 18 - 24 hours.

First Rehydration

We carefully suctioned off the C/M solution and then added 2 - 3 ml of a 50% methanol solution (50% methanol/50% distilled [ddi] water) to each tube, which were left at 20° C for 2 hours. Afterwards we carefully suctioned off the methanol and water solution.

Acid Hydrolysis

To each tube we added, 1 ml of nitrogen flushed 6N hydrochloric acid per 10 mg of skin. Next, we cooked the tubes for 18 hours in a 110° C oven, cooled them, and placed them in a Speed-Vac centrifuge dryer (Savant Instruments, Farmingdale, New York) set at continuous run/high temperature until all the acid evaporated.

Second Rehydration

We added ddi water (500 µl) to each tube to reconstitute the sample, which was then vortexed. We placed this solution in a 2 ml Costar Spin-X centrifuge tube filter (Corning Costar, Cambridge, Massachusetts) and centrifuged at 10,000 rpm for 10 – 13 minutes. We removed the tubes from the centrifuge and stored them in the freezer at approximately -10° C until required for hydroxyproline or HPLC analysis.

Hydroxyproline Assay

We estimated collagen content in the skin samples using a modified Stegemann and Stalder (1967) procedure. We assumed that hydroxyproline makes up 14% of the total collagen (Maekawa et al. 1970).

We made fresh color reagent solution (Appendix A, Table 2) the day hydroxyproline assays were run, along with a standard curve of hydroxyproline samples (n = 8) with a concentration range from 0 to 40 µg collagen/ml water. We added samples (10 µl) from the second rehydration to 990 µl of ddi H_2O and vortexed the solution for 5 - 10 seconds. We added

1 ml of Buffer B (Appendix A, Table 5) to both the standard curve and sample tubes and vortexed. Afterwards, we added 1 ml of Chloramine T solution (Appendix A, Table 1) to all tubes and vortexed them immediately after addition of the solution. We let the tubes incubate at 20° C for 10 minutes. At this time we added 1 ml of color reagent solution to each tube, followed immediately by vortexing and capping. We placed the tubes in a 60° C water bath for 20 minutes, followed by transfer to a cold water bath for 5 minutes. We then allowed the samples to incubate at 20° C for 90 minutes.

After incubation, we determined absorbency readings, in duplicate, on the spectrophotometer set at a 564 nm wavelength. If any absorbency reading was > 3.0, we diluted the sample (i.e., 8 µl sample to 992 µl water) and prepared them again for hydroxyproline analysis.

HPLC Assay

We estimated Ps concentration by reverse phase high performance liquid chromatography (HPLC) (Iqbal et al. 1997). We prepared samples in duplicate for HPLC analysis. One tube contained a mixture of sample plus a pentosidine spike (6.68 pmol/mg collagen). We required the spiked sample to ensure identification of Ps peaks. We created a standard curve ($n = 7$) ranging from 0.21 to 26.70 pmol/mg collagen.

We measured Ps concentrations using a Waters 2690 (later upgraded to a Waters 2695) HPLC separation module workstation (Waters Corporation, Milford, Massachusetts) with a Waters 474 in-line fluorescence detector (excitation: 310 nm, emission: 385 nm) (Waters Corporation, Milford, Massachusetts). We obtained elution off the C-18 column (YMC OCS-AQ 4.6 x 250 mm) using a linear gradient of 5 - 28% acetonitrile in water with 0.01 M heptafluorobutyric acid from 0 to 35 minutes. We cleaned and equilibrated the column for 18

and 12 minutes, respectively. We integrated peaks using the Millennium 32, version 3.05.01 software package (later upgraded to Empower 2 software) (Waters Corporation, Milford, Massachusetts).

Bird Age Estimates

We calculated age estimates for vultures using the double-crested cormorant age curve developed from breast skin (Fallon et al. 2006*a*). Double-crested cormorants are comparable in size to vultures (69 cm long with a 127 cm wingspan and 56 cm long and 137 cm wingspan respectively; Cornell Lab of Ornithology [CLO] 2007) and have approximately the same life span (22 years and 25 years respectively; U.S. Geological Survey [USGS] 2007). The double-crested cormorant age curve has the logistic equation: $y = 0.1914x + 6.6701$ ($R^2 = 0.93$), in which y = Ps concentration and x = estimated age in months (Fallon et al. 2006*a*). In addition, we estimated the vultures' ages using the general wild bird curve. The logistic equation for this age curve was: $y = 0.2047x + 7.4725$ ($R^2 = 0.73$) (Chaney et al. 2003). We calculated age estimates for the breast data and the patagial data.

We determined age estimates for parakeets using the wild bird age curve (Chaney et al. 2003). We only used the wild bird curve because parakeets are different in size from cormorants (28 cm long with a 48 cm wingspan; National Wildlife Federation [NWF] 2007 v.s. 69 cm long with a 127 cm wingspan; CLO 2007) and do not have the same longevity as cormorants (6 years in the wild for parakeets [Spreyer and Bucher 1998, Pruett-Jones et al. 2007] vs. 22 years in the wild for double-crested cormorants [USGS 2007]). Forty-one parakeets had either known-ages ($n = 17$) or were held in captivity for a specified amount of time, where they were at least a minimum age ($n = 24$). We then 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 determined that tests were significant using an α value of 0.05. There was a missing value for the Ps concentration from the breast skin of vulture 10 and vulture number 5 was determined to be an outlier (by regression analysis using SAS version 9.1 [SAS Institute, Cary, North Carolina]), so we removed them from all analyses.

We ran a paired *t*-test with SAS to determine if there were any significant differences in Ps concentrations between the breast and patagium. Our dependent variable was the Ps concentration and the independent variable was the body part. We tested data for normality by evaluating box plots ($g_1 = -0.22$ [vultures], $g_1 = -0.17$ [parakeets]) and homogeneity of variances by Bartlett's test for homogeneity ($\chi^2 = 0.17$ [vultures], $\chi^2 = 2.87$ [parakeets]). Data met these 2 assumptions so we did not transform data. We hypothesized that the true mean difference in Ps concentration between paired observations equaled zero.

We also used a paired *t*-test to compare estimated age for vultures and parakeets. For vultures we completed 2 comparisons: 1 using age estimates determined from the double-crested cormorant age curve and 1 using age estimates determined from the wild bird age curve. For parakeets we only used the wild bird curve for the estimated age comparison. Our dependent variable was the value for the age and the independent variable was the source of the ages (known or estimated). We tested data for normality by evaluating box plots ($g_1 = 0.25$ [cormorant curve for vultures], $g_1 = -0.22$ [wild bird curve for vultures], $g_1 = -0.17$ [parakeets]) and homogeneity of variances by Bartlett's test for homogeneity ($\chi^2 = 0.17$ [cormorant curve for vultures], $\chi^2 = 0.17$ [wild bird curve for vultures], $\chi^2 = 2.87$ [parakeets]). Data met these 2 assumptions so we did not transform data. We hypothesized that the true mean difference in age between paired observations equaled zero.

We determined the accuracy of the age estimates for vultures by comparing them to the estimated age determined from physical characteristics. Biologists with the USDA categorized each vulture as juvenile, sub-adult, or adult based on the amount of feathering (adults have fewer feathers) and the amount of wrinkles on the head (adults have more wrinkles) (CLO 2007). We confirmed the accuracy of the estimated ages for parakeets by comparing the estimated ages to the known (n = 17) and minimum (n = 24) ages for 41 parakeets.

RESULTS

Black Vultures

Vulture Ps concentrations were similar between breast ($\bar{x}$ = 8.9 pmol/mg collagen, SE = 0.55) and patagial ($\bar{x}$ = 8.9 pmol/mg collagen, SE = 0.51) skin samples (n = 28, t_{27} = 0.04, P = 0.967). The pentosidine concentrations varied for each individual vulture (Figure 1). Some individuals had concentration values close to each other for each body part (e.g., vulture 7) while others had some discrepancy in their calculated concentrations (e.g., vulture 2). The Ps concentration was higher in the patagium than the breast for 61% of the birds (n = 28).

Using the cormorant curve, no significant differences were found when age estimates were compared between breast ($\bar{x}$ = 11.6 months, SE = 2.85) and patagial ($\bar{x}$ = 11.9, SE = 2.64) data for individual vultures (n = 28, t_{27} = -0.09, P = 0.932). The breast ($\bar{x}$ = 7.0 months, SE = 2.70) and patagial ($\bar{x}$ = 7.1 months, SE = 2.49) skins produced similar estimated ages when using the wild bird curve (n = 28, t_{27}= -0.04, P = 0.969). The estimated ages for the vultures from the double-crested cormorant age curve ranged from 0 to 41 months for breast skins and 0 to 35 months for the patagial skins, while the wild bird curve produced estimated ages as 0 to 35 months for breast skins and 0 to 29 months for patagial skins (Appendix B, Table 1). Birds with age estimates less than 0 months were assumed to actually be <1 – 6 months old. Based on

physical characteristics alone, the age estimates for the vultures were 78% accurate (i.e., age estimate within 6 months of age class [juvenile, sub-adult, adult]) when compared to the age estimates from the cormorant and wild bird curves.

Monk Parakeets

Pentosidine concentrations varied between the breast ($\bar{x}$ = 15.9 pmol/mg collagen, SE = 1.30) and patagial ($\bar{x}$ = 11.5 pmol/mg collagen, SE = 1.10) skin samples for parakeets (n = 105, t_{104} = -5.14, P <0.0001) (Figure 2). Again, some individuals had concentration values close to each other (e.g., parakeet 1) while there was greater discrepancy for others (e.g., parakeet 101). Unlike the vultures, however, the parakeets more often had higher Ps concentrations in the breast (72% of the samples, n = 105).

The results indicate that there were significant differences when age estimates were compared between breast ($\bar{x}$ = 41.0 months, SE = 6.34) and patagial ($\bar{x}$ = 19.8 months, SE = 5.37) data for individual parakeets (n = 105, t_{104} = -5.16, P <0.0001). The estimated ages for the parakeets from the wild bird age curve ranged from 0 to 255 months for breast skins and 0 to 191 months for the patagial skins (Appendix C, Table 1). Birds with age estimates less than 0 months were assumed to actually be <1 – 6 months old.

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 that had a minimum age had age estimates higher than the length of time they spent in captivity (Appendix C, Table 1). 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 that had a minimum age had age estimates higher than the length of time they spent in captivity (the other 2 birds had age estimates only 2 and 12 months lower than their captive holding time).

DISCUSSION

This study confirmed previous research studies on Ps accumulation with age. Similar to ruffed grouse, double-crested cormorants (Fallon et al. 2000*a*), wild birds (Chaney et al. 2003), and chickens (Iqbal et al. 1999), pentosidine was found to accumulate with age in parakeets.

The data from these studies suggest that an age curve generated from patagial skin of vultures could reliably estimate the age of a vulture from a skin sample taken from the breast. This was confirmed when the age estimations were compared from patagial and breast skin. If live sampling of vultures becomes a wildlife tool then the patagium will most likely be the location to obtain a skin sample.

Both the cormorant and wild bird curve produced similar age estimates, suggesting that either one of these curves will adequately estimate an accurate age for vultures. When comparing age estimates based on physical characteristics to the ones determined from the age curves (Appendix B, Table 1), we find that with the exception of vultures 2 and 8, these age estimates appear to correspond. Vulture 2 had an age estimation of less than 1 year old and vulture 8 had an age estimate of 11-20 months old using Ps analysis. Despite placing the birds in a different age class, we find that the age estimations were only off by several months.

The parakeet study produced similar results to the Fallon et al. (2006*b*) findings for Ps concentration differences, although in the parakeet study the Ps concentration was higher in the breast. These data suggest that known-age curves for parakeets will need to be developed for specific body parts. One explanation as to why Ps 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 often fly, whereas ruffed grouse, are mainly land-based birds and seldom fly, except for short distances. We argue that more oxidative stress occurs in the flight muscles of

parakeets. This argument can be expanded to include the vultures. Vultures also fly a lot, but they often glide on air currents and soar during migration, which requires little flapping with their wings (Rappole 2006), thus not relying on their flight muscles as much. Because ruffed grouse don't often fly and vultures glide and soar, oxidative stress does not occur as much in their breast muscles. Oxidative stress has been found to increase for muscles that go through excessive exercise (Ji 1999). Advanced glycation endproducts, such as Ps, are highly associated with oxidative stress in birds (Iqbal et al. 1999, Klandorf et al. 1999). A future study could examine breast skin of migratory and non-migratory birds of the same species to see if the level of use of flight muscles (i.e., oxidative stress) could influence the concentration of Ps in skin.

The differences in parakeet age estimations can be attributed to the differences in Ps concentrations for 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 despite a significant difference in estimated age, both estimated ages could still be accurate to within a reasonable period. For example, parakeet H18 had an age estimate of 1 month from the breast skin and 7 months from the patagial skin, but the true age was 2 months. Because of the high accuracy of estimated ages (i.e., within 6 months) for both body parts of parakeets, this may be one of the cases where significant findings (i.e., significant differences between Ps concentrations and estimated ages) may not be biologically important.

The variance in Ps concentrations for vultures and parakeets may occur based on their taxonomy. Parakeets and vultures are completely different species of birds, in different taxonomic orders (order Psittaciformes for parakeets and order Ciconiiformes for vultures [Rappole 2006]). Further investigations will need to take place to see if differences are more pronounced based on genus, family, or order or if it occurs randomly by individual species.

Pentosidine differences also may occur based on oxidative stress. There may be differences in vasculature of the body regions of vultures and parakeets. If one region contains more capillaries, it will become perfused with blood, which may cause more oxidative stress, resulting in higher Ps concentrations.

MANAGEMENT IMPLICATIONS

The logical next step would be to apply the measurement of Ps in living birds. Consideration for their health and welfare must be of utmost importance. Avian anatomy literature states that the breast is a more sensitive area in regards to flight, than the wing. Because this study indicates that Ps concentrations are generally the same for the breast and patagium of birds, it is our recommendation that skin samples be collected from the patagium of living birds. However, if there truly is a difference in Ps concentration for parakeets, then a patagial skin curve will need to be developed for this species once older known-aged individuals become available. For vultures, either skin location can be used to create an age curve.

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FIGURES

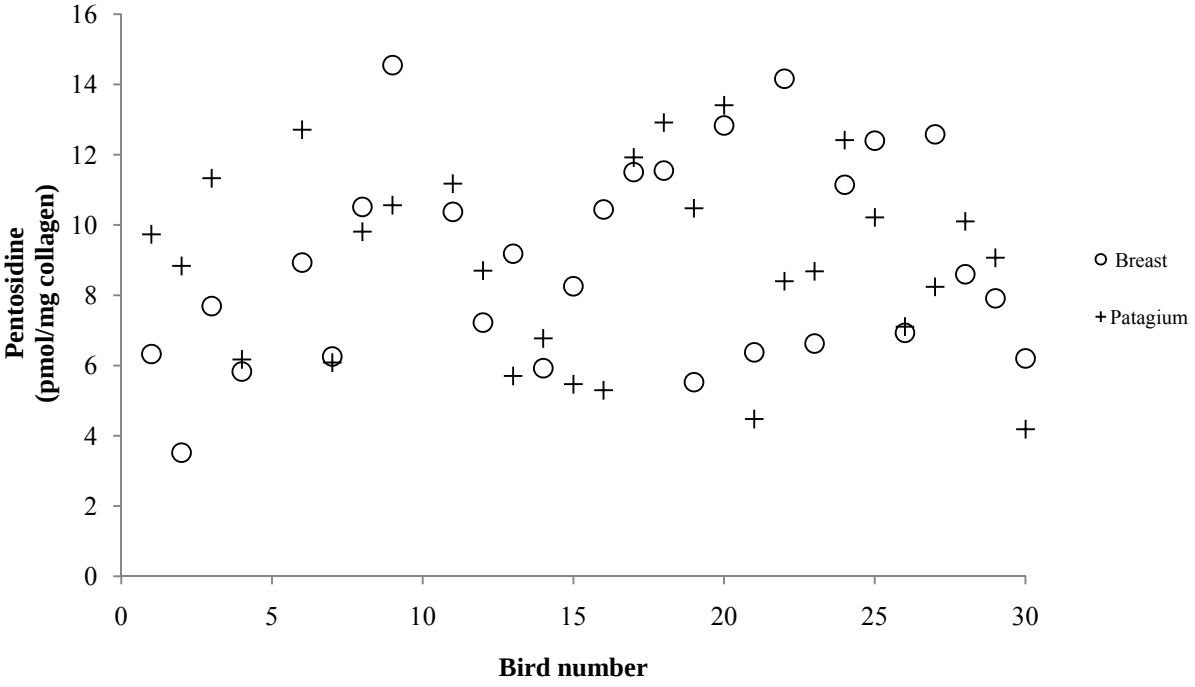

Figure 1. Distribution of pentosidine (Ps) concentrations for patagial and breast skin of 28 black vultures from Gainesville Florida, USA. Pentosidine concentrations were higher in the patagium more often than the breast. The majority (89%) of the vultures have Ps concentrations within 5 pmol Ps/mg collagen for both locations.

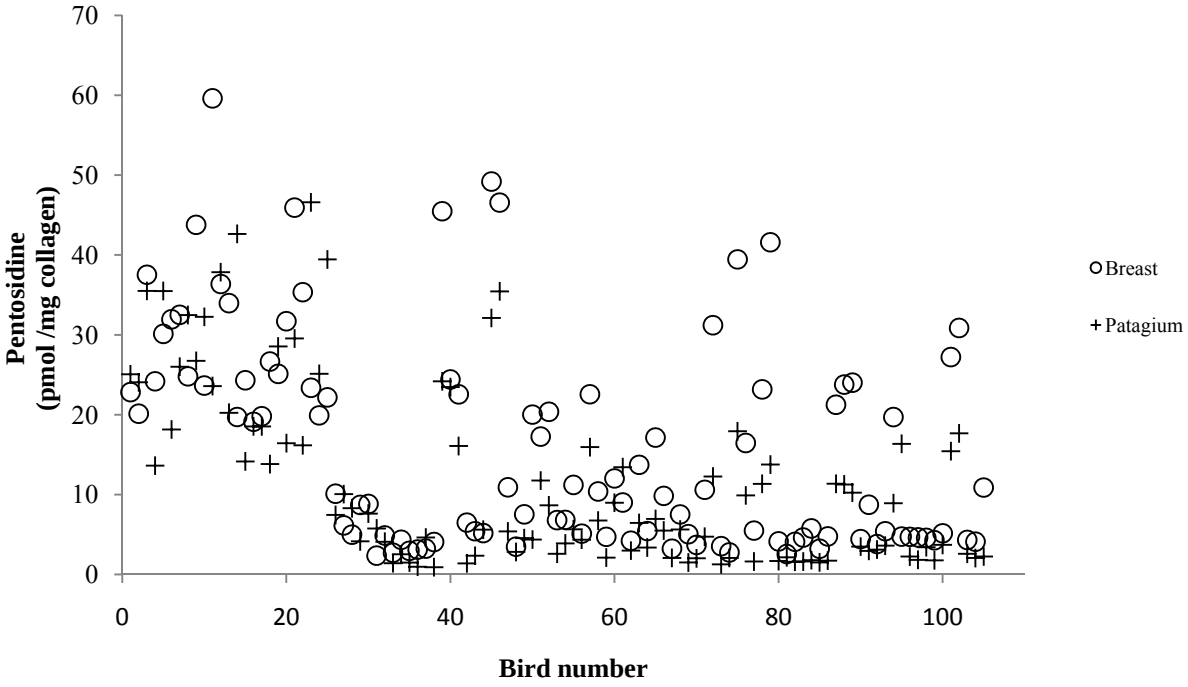

Figure 2. Distribution of pentosidine (Ps) concentrations for breast and patagial skin of 105 monk parakeets from Gainesville Florida, USA. Pentosidine concentrations were higher in the breast more often than the patagium. The majority (72%) of the parakeets have Ps concentrations within 10 pmol Ps/mg collagen for both locations.

CHAPTER III

An *In Vivo* Pentosidine Aging Technique Evaluation for Double-crested Cormorants

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RH: Cooey et al. • Live Bird Aging Technique Evaluation

An *In Vivo* Pentosidine Aging Technique Evaluation for Double-crested Cormorants

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ABSTRACT A live sampling protocol for birds to age them by measuring pentosidine (Ps) concentration in the skin has never been attempted. Considerations include the location of the skin biopsy, the amount of skin to process, and the closure method of the wound. We investigated the development of a minimally invasive protocol for live sampling birds as well as the results of using this protocol in a live sampling study. Double-crested cormorants (*Phalacrocorax auritus*) are an optimal species to study because of the need to understand age demographics of this species due to their real or perceived impacts to commercial and natural resources. The patagium from known-aged cormorants (n = 63) was selected as the preferable location to take skin biopsies, although the standard age curve for cormorants was created with data from breast skin. Comparison of Ps concentrations between the patagium ($\bar{x}$ = 10.6 pmol/mg collagen, SE = 1.10) and the breast ($\bar{x}$ = 11.2 pmol/mg collagen, SE = 1.10) of known-

This chapter is written in the style of *The Journal of Wildlife Management*

aged cormorants revealed that there were no significant differences between those locations (P = 0.10). Pentosidine concentration was marginally higher in 6 mm^2 ($\bar{x}$ = 12.6 pmol/mg collagen, SE = 1.19) than the 20 mm^2 ($\bar{x}$ = 11.3 pmol/mg collagen, SE = 1.23) patagial skin samples (P = 0.02). In addition, new age curves (our breast skin age curve and our patagial skin age curve) were developed from known-aged cormorants (n = 58) and compared to the age curve used in a previous study (Fallon age curve). Our age curves were more accurate in predicting age and were determined to estimate age within 14 - 30 months of the actual age. In a seperate study, we took 6 mm^2 biopsy samples *in vivo* from both the breast and patagium of 7 wild-caught cormorants (3 juveniles, 4 adults) from Bluff Lake, Noxubee National Wildlife Refuge in Mississippi. Wounds were closed with dissolvable sutures for 4 birds while the remaining 3 were closed with tissue glue. No significant differences occurred between the Ps concentrations of the breast ($\bar{x}$ = 14.7 pmol/mg collagen, SE = 2.70) and patagium ($\bar{x}$ = 12.2 pmol/mg collagen, SE = 1.82) of the living cormorants (P = 0.20). Healing time was similar between the breast ($\bar{x}$ = 15.9 days, SE = 1.36) and patagium ($\bar{x}$ = 15.8 days, SE = 1.85) (P = 0.79) but the wounds closed with tissue glue ($\bar{x}$ = 14.5 days, SE = 1.12) healed significantly faster than those closed with sutures ($\bar{x}$ = 17.3 days, SE = 0.66) (P = 0.0003). These cormorants' ages were estimated using the same age curves, and the accuracy of the curves were determined using known-age data (n = 1) and juveniles identified through plumage coloration and lack of sexually mature reproductive organs (n = 4). In this analysis the Fallon age curve had the most accurate age estimate for the known aged cormorant. Live sampling birds for Ps analysis is a viable technique and estimates age with a 4.5 year confidence limit, suggesting that this technique can be useful for aging long-lived birds. We recommend taking 6 mm^2 skin samples from the patagium and the wounds closed with tissue glue.

KEY WORDS avian aging, biopsy punch, breast skin, double-crested cormorant, live sampling, patagium, pentosidine, *Phalacrocorax auritus*, skin size, sutures, tissue glue, wound closure.

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The past 3 decades have seen a population resurgence of double-crested cormorants (*Phalacrocorax auritus*, hereafter cormorants) in North America, with doubling-times of < 5 years for cormorants in some areas (Hatch and Weseloh 1999, Glahn et al. 2000*a*, Taylor and Dorr 2003). Increases in cormorant numbers have resulted in both perceived and documented impacts to recreational fisheries (Taylor and Dorr 2003, Rudstam et al. 2004), sensitive vegetation (Hebert et al. 2005), other colonial-nesting birds (Jarvie et al. 1999), and channel catfish (*Ictalurus punctatus*) aquaculture (Wywialowski 1999, Glahn et al. 2000*b*, Glahn and Dorr 2002, Glahn et al. 2002).

Increased numbers of cormorants, particularly for the interior population, concomitant with increased concerns over possible negative impacts to commercial and natural resources have resulted in management efforts aimed at mitigating those impacts (Taylor and Dorr 2003). Management practices, such as harassment, have proven difficult due to regional increases in cormorant numbers and because they provide only a temporary and local reduction in cormorant numbers (Mott and Boyd 1995, Glahn et al. 2000*b*). In response to the perceived declining effectiveness of these programs, various local- and regional-level depredation management efforts were initiated (Dorr et al. 2008). These efforts include establishment of the 1998 Depredation Order specific to aquaculture (U. S. Fish and Wildlife Service [USFWS] 1998) and in 2003, establishment of a Public Resource Depredation Order, which allows various agencies to conduct cormorant control, including lethal take, on affected natural resources in 24 states including Mississippi (USFWS 2003, Dorr et al. 2008). This regulatory movement towards

cormorant management including lethal control has resulted in an acknowledged gap in basic understanding of how management may affect the interior cormorant population. Knowing age demographics for this species is vital for modeling and linking of management information to demographic response of the cormorant population (Frederiksen and Bregnballe 2000, Glahn et al. 2000*a*).

Analysis of pentosidine (Ps) concentrations in the skin has been identified as an aging tool for deceased birds (Iqbal et al. 1999, Chaney et al. 2003, Fallon et al. 2006*a*), including those that have been prepared as museum mounts (Fallon et al. 2006*b*). However, before age estimates from Ps analysis becomes a viable aging tool for living birds, a minimally invasive technique needs to be developed. Previous Ps age curves were developed from breast skin, however, the breast may not be the best location to take a skin sample from living birds. If the biopsy punch is pressed too hard, the pectoralis major muscle may be damaged and reduce flight capability (Proctor and Lynch 1993). For this reason, the patagium on the wing of the bird may be a better location to sample live birds. Fewer muscles in the wing (Proctor and Lynch 1993) and visibilty of veins in the patagium allows for skin collection to occur with minimal bleeding (Smallwood and Natale 1998). By avoiding the veins, there is also a smaller chance of infection reaching the blood stream, thereby minimizing any complications associated with the sampling procedure (Beal et al. 2000, Muza et al. 2000).

Other considerations when working with the breast and patagium are the concentration of Ps in those body parts and the size of the skin sample needed. Fallon et al. (2006*b*) determined that Ps concentrations varied between skin locations of ruffed grouse (*Bonasa umbellus*). In ruffed grouse, Ps concentrations were significantly greater in the patagium than the breast (Fallon et al. 2006*b*). In another study, when comparing 4 mm^2, 6 mm^2, 8 mm^2, and 20 mm^2

skin sizes from black vultures (*Coragyps atratus*), no significant differences in Ps concentration was found to occur (J. A. Fallon, West Virginia University, unpublished data). Skin biopsies from birds can be taken with readily available 6 mm diameter biopsy punches (Nett et al. 2003). Minimizing the area of skin samples reduces invasiveness. To age living cormorants with a skin sample taken from the patagium, it must be determined if Ps concentrations vary between the breast and patagium, and if there are differences between 6 mm^2 and 20 mm^2 skin samples.

Finally, the most effective wound closure method should be employed when sampling live birds. Surgical glue (tissue glue) and dissolvable sutures are 2 closure methods that have been successfully used to close wounds on birds (Small et al. 2004, Schwagmeyer et al. 2005). When tissue glue is applied to a wound site as a liquid, it polymerizes to a firm, pliable film that binds to the skin and holds the edges of the wound together (Hollander and Singer 1999). Comparatively, sutures close dead space, support and strengthen the wound until healing increases its tensile strength, and minimize the risk of bleeding and infection (MacKay-Wiggan and Ratner 2007). The simple interrupted suture stitch, the most commonly used stitch in cutaneous surgery, optimally distributes tension on the wound, achieves eversion, and has less potential for causing wound edema and impaired cutaneous circulation, although it takes longer to complete in comparison to other suture techniques (MacKay-Wiggan and Ratner 2007). Tissue glue is faster to apply than sutures (Trott 1997), but no literature has been found that indicates which of these methods is more effective in allowing the wound to heal.

The objectives of our studies are to 1) compare Ps concentrations from the breast and patagium of known-age cormorants, 2) compare Ps concentrations between 6 mm^2 and 20 mm^2 skin sizes, and 3) test the efficacy of a minimally invasive sampling protocol on 7 living cormorants. Based on the results of Fallon et al. (2006*b*), we hypothesize that there will be a

significant difference in Ps concentrations between the breast and patagial skin. Based on the results of the skin size study with black vultures (J. A. Fallon, West Virginia University, unpublished data), we predict that there will be no significant differences in Ps concentrations for these 2 skin sizes. Finally, we hypothesize that there will be no significant difference in healing rate between sample sites as a function of the closure method.

STUDY AREA

Banded cormorants were lethally collected from their wintering grounds in Mississippi, western Alabama, and eastern Arkansas, and from their breeding grounds on Lake Huron and Lake Michigan in the upper peninsula of Michigan from November 1999 to March 2007. Live cormorants were captured from Bluff Lake in Noxubee National Wildlife Refuge in east-central Mississippi, about 22 km south of Starkville, MS in January 2008. The 19,500 ha refuge was established in 1940 and serves as a resting and feeding area for migratory birds (U.S. Fish and Wildlife Service [USFWS] 2008). Bluff Lake is a 324 ha lake (USFWS 2008) with vegetation including bald cypress (*Taxodium distichum*), water tupelo (*Nyssa aquatica*), and buttonbush (*Cephalanthus occidentalis*).

METHODS

Skin samples from 63 deceased, known-age cormorants were procured at necropsy at the National Wildlife Research Center's (NWRC) Mississippi Field Station. Cormorants were banded as juveniles, and their ages were calculated using banding records from the USGS Bird Banding Laboratory. Approximately 100 mg of breast and patagial skin from each cormorant was procured at necropsy. All skins were placed in plastic vials containing distilled water. The containers were frozen and shipped overnight to West Virginia University (WVU) in Morgantown, West Virginia, USA for Ps analysis.

Study 1: Breast and Patagium Ps Concentration Comparison

We prepared samples (20 mm^2 or about 40 mg) from both the breast and patagial skins for each known-age cormorant for analysis. We processed samples as described by Iqbal et al. (1999). Briefly, this process involved skin cleaning, delipidation, rehydration, acid hydrolysis, and a second rehydration. We determined collagen content through spectrophotometric hydroxyproline analysis using a DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA) with a 564 wavelength, assuming 14% of collagen to be hydroxyproline (Maekawa et al. 1970). We measured Ps concentrations through reverse-phase High Performance Liquid Chromatography (HPLC). We analyzed Ps samples in duplicate, where one sample was spiked with a Ps standard to determine elution time. Integration of peaks was done with Millennium 32, version 3.05.01 software (Waters Corporation, Milford, MA) (later upgraded to Empower 2 software [Waters Corporation, Milford, MA]).

Cormorant Age Curves. – We developed 2 age curves for the cormorants using the age data obtained from banding records and the Ps concentrations measured for the patagium and breast skin (n = 58). We developed a breast skin age curve (hereafter our breast skin age curve) and a patagial skin age curve (hereafter our patagial skin age curve). We fit the age curves with a linear and curvilinear (power) regression line.

Actual and Estimated Age Comparison. – We determined actual ages for the deceased cormorants based on band records (n = 63). We then calculated an estimated age for each cormorant using the linear and curvilinear regression equations from our breast and our patagial skin age curves. We calculated 2 estimated ages per cormorant (1 for the breast skin and 1 for the patagial skin). We also calculated an estimated age for the breast and patagial skins for each

cormorant using the cormorant age curve generated by Fallon et al. (2006*a*, hereafter Fallon age curve).

Study 2: Skin Size Ps Concentration Comparison

We used skins from 50 of the same 63 cormorants in study 1 to compare Ps concentrations between 6 mm^2 and 20 mm^2 skin sizes. We used a 6 mm diameter disposable biopsy punch (Miltex Inc., York, PA) to take a skin sample from the patagial skins of each bird. We compared the Ps concentration from these samples to the 20 mm^2 patagial Ps concentrations determined in Study 1.

Comparison of Estimated Ages for Different Skin Sizes. – Because skin samples were taken from the patagium only, we estimated ages for the 50 cormorants using our linear and curvilinear patagial skin age curve.

Actual and Estimated Age Comparison. – After obtaining estimated ages from the 2 different sized skins using our linear and curvilinear patagial skin age curves, we compared actual and estimated ages.

Study 3: Live Cormorant Sampling

Cormorant Capture. – We traveled to Bluff Lake in the Noxubee National Wildlife Refuge in January 2008 for cormorant capture. We captured cormorants using the method as described by King et al. (1994). We traveled in pairs 30 minutes after sunset, via motorized jon-boats (4.6 m length, 0.6 m high bow rail, padded bow seat for kneeling, 25-hp outboard, and 3 500-W floodlights). One of us piloted the boat and scanned the water for cormorants with a spotlight. The other sat at the bow of the boat with a large dip net (0.8 m diameter and 1.2 m deep with a 2-3 m handle).

When a large group or "raft" of cormorants was found on the surface of the water, we blinded the cormorants with the floodlights to disorient them. As the boat approached the birds, we attempted to capture a cormorant with the dip net before it flew away or dove beneath the water's surface. In all, we captured 7 cormorants and transported them by vehicle to the USDA NWRC Mississippi Field Station aviary located adjacent to Mississippi State University in Starkville, MS.

Holding Facilities and Quarantine. – We weighed the birds the same night of capture. Cormorants were individually housed in pens (304.8 cm x 304.8 cm x 182.9 cm) with 200 L diving pools. The pools were equipped with ramps for perching and the birds were provided continuous accessibility to water with a recirculating water system containing bio-filters and particulate waste filters to maintain water quality.

We held the cormorants in quarantine for 2 weeks before skin samples were taken. During this time, we monitored birds for any signs of illness, disease, or severe stress that would exclude them from the study. Cormorants were fed an ad-libitum diet of between 600-800 g of live channel catfish fingerlings daily. We provided fish to each bird in their respective 200 L diving pools. We removed uneaten catfish from the pools each day and weighed them to determine the average mass of food each individual cormorant consumes daily and cleaned the pens daily.

Skin Sample Collection. – On 5 February 2008 we collected skins using a modified technique from the U. S. Department of Agriculture (2007) with a veterinarian present to oversee the sampling procedure. We removed the cormorants from their holding pens, one at a time, and took them into the workshed within the aviary.

At the sampling site, skin was first disinfected with isopropyl alcohol. We then administered a subcutaneous injection of lidocaine (1-4 mg/kg diluted 1:10 with 0.9% Normal saline [Paul-Murphy and Ludders 2001]) to anesthetize the local area. We used a sterile, disposable 6 mm diameter biopsy punch (Miltex Inc., York, PA) to obtain a skin sample. Using a clockwise/counterclockwise rotation (Nett et al. 2003), we penetrated the skin with the biopsy punch. If the skin was still attached to sub-dermal layers, we used a pair of surgical scissors to cut away the skin sample. We then placed the skin sample in a container filled with distilled water.

After removing any blood from the sample site with sterile gauze, we closed the incision with either Vetbond Tissue Adhesive (3M, St. Paul, MN) or Coated Vicryl Violet Braided 6-0 absorbable sutures (Ethicon Inc., New Brunswich, NJ). Three of the cormorants had their wounds closed with tissue glue while the remaining 4 had them closed with dissolvable sutures. If the wound was closed with tissue glue, one of us pinched the wound so only a slit was visible. After applying tissue glue to the wound and allowing a few seconds for drying, we used forceps to secure the closure by pinching the tissue glue onto the wound. We applied more tissue glue (if needed) until the wound was completely sealed. If sutures were used to seal the wound, then we applied 2-3 sutures, using the simple interrupted stitch, to close the wound. We froze the containers holding the skin samples and then shipped them overnight to WVU for analysis.

Age Estimates. – We estimated ages for each of the living cormorants using their Ps concentration and the 5 age curves discussed in Study 1. One live-cormorant captured was banded and its age was determined from banding records. We also determined accuracy of the age estimates through Ps analysis by aging the birds by their plumage (Hatch and Weseloh 1999)

and examining gonadal development (Sherwood et al. 2005) after euthenization at the end of the study.

Wound Monitoring. - Average healing time for superficial wounds on birds is 10-14 days (Burke et al. 2002), so we inspected the wounds on days 2, 6, 9, 14, and 17 post-sampling. We took photographs at each wound check to document the healing progression (Appendix F). No infection occurred, but if it did, we would have applied a topical medication. We also weighed the cormorants at each monitoring session to determine if they lost mass post-sampling. Any mass loss or development of anorexia may be an indicator that the bird developed septicemia (Xi et al. 2007).

Statistical Analysis

We determined significant differences using an α value of 0.05. We tested data for normality by evaluating box plots for skewness and outliers (Connolly 1989) and by evaluating normal probability plots (Chambers et al. 1983), and we tested data for homogeneity of variances with Bartlett's test for homogeneity (Mudholkar et al. 1993) before analyzing the data. All data met the assumptions, so we did not transform the data. All data analyses were completed using SAS software (SAS Institute, Cary, NC).

Study 1. - We used a paired *t*-test to determine if there were any significant differences in Ps concentrations between the breast and patagium of the known-age cormorants. We used Studentized residuals (cut off values < -2 and > 2) to determine outliers for the actual ages for the Ps data (Cook 1982). We omitted all outliers when developing a known-age cormorant curve. We also used a paired *t*-test to determine if there were significant differences between actual and estimated ages for the known-age cormorants using all 5 age curves and used an upper

and lower 95% Confidence Limit around the mean estimated age to determine the accuracy of the estimated ages.

Study 2. –We used a paired *t*-test to determine if there were any significant differences in Ps concentrations between the 2 skin sizes of known-aged cormorants. We also used a paired *t*-test to determine if there were any significant differences between estimated ages for each skin size of the cormorants. We determined the accuracy of the age estimates by producing upper and lower 95% Confidence Limits around the mean estimated age.

Study 3. - We ran a paired *t*-test to determine if there were any significant differences in Ps concentrations between the breast and patagium samples from living cormorants, in estimated ages determined for each cormorant from the sampling site data, in healing rates between locations, and in healing rates between closure methods. We also used a 2-way analysis of variance (ANOVA) to determine if the effect of closure method on healing rate was influenced by the sampling site location. The dependent variable was the healing time and the independent variables were the body part and the closure method.

RESULTS

Study 1: Breast and Patagium Ps Concentration Comparison

Pentosidine concentrations sampled from each location varied for each individual cormorant (Figure 1). Concentrations of Ps were not significantly different ($t_{62} = 1.66$, $P = 0.10$) in the breast ($\bar{x}$ = 11.2 pmol/mg collagen, SE = 1.1) and patagium ($\bar{x}$ = 10.6 pmol/mg collagen, SE = 1.1) of cormorants. However, for 65% of the samples ($n = 63$), the Ps concentration was greater in the breast.

A curvilinear regression line provided the best fit for both the breast (Figure 2, R^2 = 0.7806) and patagial skins (Figure 3, $R^2 = 0.8164$). Both of these curves suggest that the

cormorants accumulate Ps quickly when they are young, and then begin to accumulate Ps at slower rates once they reach the age of approximately 25 months. The mean estimated age using our breast skin age curve produced an age estimate of 73.5 months ± 17.4 months, while the mean estimated age using our patagial skin age curve was 62.4 months ± 13.5 months.

The linear regression equations for our breast ($y = 0.0973x + 4.8997$, $R^2 = 0.7108$; Figure 2) and our patagial age curves ($y = 0.1005x + 4.1671$, $R^2 = 0.7575$; Figure 3) were similar. The age curves from our breast and our patagial skins indicated that cormorants accumulate Ps at a rate of approximately 0.10 (pmol/mg collagen)/month. The mean estimated age using our breast skin age curve was 62.0 months ± 15.1 months, while the mean estimated age from our patagial skin age curve was 67.2 months ± 14.7 months.

For the breast and patagial skins of the 63 known-age cormorants, the ages estimated using our curvilinear patagial skin age curve were most accurate (i.e., the closest age estimate from all 5 age curves to the actual age) for 33% of the samples (breast and patagial samples combined, Appendix D). For example, cormorant 42 was actually 14 months old when it died; the age estimate for cormorant 42's breast skin from the Fallon age curve was -8 months (22 month difference), our linear breast skin age curve was 3 months (11 month difference), our linear patagial skin age curve was 10 months (4 month difference), our curvilinear breast skin age curve was 11 months (3 month difference), and our curvilinear patagial skin age curve was 14 months (0 month difference). Our linear breast skin age curve produced the least number of closest age estimates to real ages (11% of samples).

There were significant differences in actual and estimated ages for breast and patagial skins of the known-age cormorants when using the Fallon age curve to estimate ages (Table 1).

However, actual and estimated ages were similar when using our linear and curvilinear breast and patagial skin age curves to age the cormorants.

Study 2: Skin Size Ps Concentration Comparison

Pentosidine concentrations varied for each individual cormorant when the 2 different skin sizes were compared (Figure 4). Generally most individuals had concentration values close to each other for each skin sample (i.e., cormorant 1), although, for 64% of the samples the Ps concentration was higher in 6 mm^2 skin size sample than in the 20 mm^2 sample. For 84% of the samples, the Ps concentration difference between the 2 skin sizes was less than 5 pmol Ps/mg collagen. For only 2 samples, the difference between skin size Ps concentrations was over 10 pmol Ps/mg collagen, which likely reflects a methodological error. Pentosidine concentrations were marginally higher in 6 mm^2 ($\overline{x}$ = 12.6 pmol/mg collagen, SE = 1.19) skins compared to 20 mm^2 ($\overline{x}$ =11.3 pmol/mg collagen, SE = 1.23) skins (t_{49} = -2.42, P = 0.02).

Mean estimated ages for 6 mm^2 skins using our linear and curvilinear patagial skin age curves were $\overline{x}$ = 83.5 months ± 23.8 months and $\overline{x}$ = 90.0 months ± 27.5 months respectively. Similarly, the mean estimated ages for 20 mm^2 skins using the same linear and curvilinear curves were $\overline{x}$ = 70.6 months ± 24.6 months and $\overline{x}$ = 79.6 months ± 28.3 months respectively. There was a marginal significant difference between the real and estimated ages determined from 6 mm^2 skins using our curvilinear patagial skin age curve, but real and estimated ages were similar for the 6 mm^2 skins using our linear patagial skin age curve as well as the 20 mm^2 skins using our linear and curvilinear patagial skin age curves (Table 2).

Study 3: Live Cormorant Sampling

The tissue glue took approximately 1 minute to adequately seal the biopsy site. The sutures took approximately 3-4 minutes to close the wounds of the cormorants.

Pentosidine concentrations varied for each individual living cormorant when 2 different body parts were compared (Figure 5). Most individuals had concentration values within 3 pmol Ps/mg collagen for each body part (e.g., cormorant 2) while 1 had a concentration difference of 12.5 pmol Ps/mg collagen (i.e., cormorant 1). For 71% of the birds, the Ps concentration was higher in breast skin. No significant difference was found when the Ps concentrations were compared between the breast ($\bar{x}$ = 14.7 pmol/mg collagen, SE = 2.70) and patagium ($\bar{x}$ = 12.2 pmol/mg collagen, SE = 1.82) of the living cormorants (t_6 = -1.43, P = 0.20).

Cormorant AJL was 32 months old at the time of capture, and the Fallon age curve was most accurate in estimating its age through Ps analysis of the patagial skin (estimate of 30 months, Table 3). The Fallon and our linear patagial skin age curves generally produced the youngest and oldest age estimates, respectively. The youngest and oldest cormorants appear to be birds 18 and 86, respectively, based on all 5 age curve age estimations.

No significant difference occurred for the sample site × closure method interaction (F_1 = 2.14, P = 0.17). This indicates that the effect of the closure method does not depend on the body part. Healing time was significantly shorter for wounds closed with tissue glue ($\bar{x}$ = 14.5 days, SE = 1.12) compared to sutures ($\bar{x}$ = 17.3 days, SE = 0.66) (F_1 = 26.10, P = 0.0003). There was no significant difference in healing time for the breast ($\bar{x}$ = 15.9 days, SE = 1.36) and patagium ($\bar{x}$ = 15.8 days, SE = 1.85) of the living cormorants (F_1 = 0.07, P = 0.79).

DISCUSSION

Study 1: Breast and Patagium Ps Concentration Comparison

Concentrations of Ps varied between locations. In order to determine if this difference is due to methodological error or location on the body, multiple skin samples could be collected, analyzed, and compared in the future.

There was no significant difference in Ps concentration between sampling sites. This finding differs from the one discovered by Fallon et al. (2006*b*), where Ps concentrations were higher in the patagium compared to the breast of ruffed grouse, a non-migratory bird. For this study, the higher Ps concentration in the breast skin of 65% of birds may be explained by the cormorant's flight habits. Cormorants are migratory birds (Hatch 1995, Werner and Dorr 2006). Because of migration they may have an increased vasculature and exposure to oxidative stress (Madamanchi et al. 2005) around their flight muscles, resulting in higher Ps concentrations in the breast skin. An experiment comparing Ps concentrations in the breast skin of migratory and non-migratory birds of the same species would need to be done to support this theory.

Both curvilinear regression lines for our breast and our patagial skin age curves had a better fit of the data than a linear regression. All previous aging analyses for cormorants have been done with the Fallon age curve, which uses a linear curve. Research by Fallon et al. (2006*a*), Chaney et al. (2003), and Iqbal et al. (1999) suggested that Ps continues to accumulate over the lifetime of the birds and never levels out. This study suggests, however, that Ps accumulates rapidly the first 2 years of life and then slows down in production rate.

Our linear and curvilinear breast and patagial skin age curves produced similar estimated ages (within several months of each other). Our curvilinear patagial skin age curve proved to be more accurate in predicting age than the original Fallon age curve. The difference between the Fallon age curve and our breast and patagium age curves may be due to differences in laboratory analyses. Also, the Fallon age curve was generated with data from 19 cormorants, whereas our breast and patagium age curves used over 3 times as many samples. On further examination of the age estimates, our curvilinear age curves were identified to be most accurate in predicting ages for younger birds, while our linear age curves were more accurate in estimating the age of

older cormorants (Appendix D). A combination of the linear and curvilinear age curves may need to be done to produce more accurate estimated ages. Until this is done, we suggest using our curvilinear age curves to estimate age in cormorants, because our data suggest that cormorants accumulate Ps more rapidly when they are young and all age estimates calculated a positive age (as opposed to a negative age estimate from our linear breast and patagium age curves).

The age estimates from our curvilinear breast and patagial age curves produced age estimates that were calculated to be accurate to within approximately 1 ½ years (17.4 months) of the actual age. An age estimate as accurate as this for long lived species, such as cormorants, could provide valuable insight on senescence, reproductive success, and behavioral changes for different adult age classes. If avian members of the Species Survival Program (SSP) could be aged to within 1½ years of their actual age, reproductive success in captive breeding programs may increase when similarly aged individuals are paired together (Fulai et al. 1995).

Study 2: Skin Size Ps Concentration Comparison

The results from this study were counterintuitive. The concentrations of Ps were marginally greater in the smaller skin size sample, concurring with the observation that Ps accumulation in the skin is not uniform (Fallon et al. 2006*b*). Depending on the network of vascularity in a particular region, the collagen may be exposed to differential oxidative stress and this accumulates Ps at slightly different rates. As a result differences in Ps concentration were found to occur between the 2 skin sizes, which in turn gave age estimates that were also different from actual ages when using our curvilinear patagial skin age curve. Our linear patagial skin age curve calculated no significant differences between actual and estimated ages for the 2 skin sizes of cormorants, which suggests that the difference in Ps concentration may not be biologically

important. Additional studies on other long-lived species need to be conducted to determine if Ps differences between skin sizes influence age estimates. The confidence limits suggest that using our breast and patagial skin age curves will produce age estimates within approximately 2½ years (28.3 months) of the actual age.

Study 3: Live Cormorant Sampling

Captive birds that had skin excised for Ps measurement did not develop an infection. Although the birds were maintained in a restricted environment, it is likely that birds released to the wild are unlikely to develop an infection as well. This indicates that both tissue glue and sutures are effective against infection development, however, research with rats (*Rattus norvegicus*) found that wounds closed with sutures had a greater chance of developing abscesses or minor inflammation when compared to wounds closed with tissue glue (Vanholder et al. 1993). Tissue glue helps prevent infection and is known to promote healing (Spotnitz et al. 1997). In a study of testosterone-regulating implants in house sparrows (*Passer domesticus*), none were reported to have developed infection or died from complications at the implantation site (Schwagmeyer et al. 2005). These birds also had their wounds closed with sutures and tissue glue (Schwagmeyer et al. 2005). We recommend that tissue glue be used to close the wounds, due to ease of use, and literature indicating that tissue glue results in fewer cases of infection.

Similar to Study 1, the Ps concentration was found to be numerically greater in the breast of living cormorants, but was not significantly different from Ps concentrations in patagial skin. The lack of a significant difference between Ps concentrations in the breast and patagium of the living cormorants supports the results obtained in Study 1. Because the data from the living cormorants was similar to the findings obtained from deceased known-age cormorants, we can make the assumption that living and deceased birds of the same species will produce similar

pentosidine results (i.e., no significant differences in Ps concentrations for body parts). However, our results should be interpreteted with caution as we had a small (n = 7) sample size for live birds.

Based on the rates of healing, the water-resistant tissue glue (Hollander and Singer 1999), allowed the wounds to heal faster. Because wounds closed with sutures should remain dry for the first 24-48 hours to prevent infection development (Heal et al. 2006, Noe and Keller 1988) this technique may limit its usefulness for aquatic birds. Cormorants released back into their holding pens were all seen swimming in their tanks within an hour of suture application. The chance of infection for the sutured birds could possibly be higher than for the cormorants with wounds closed with tissue glue, especially if there were bacteria in the water. The water exposure may have also kept the wounds wet which increased the clotting time. Birds in general have a slower clotting time in comparison to higher mammals (5-30 minutes and 1-14 minutes respectively) (Bigland and Triantaphyllopoulos 1960). If the cormorants with sutures took longer for their wounds to clot, they would not be able to begin healing at the same rate as the cormorants with tissue glue applied, thus increasing total healing time.

Black ducks (*Anas rubripes*) (Harms et al. 1997) and Florida sandhill cranes (*Grus canadensis pratensis*) (Klugman and Fuller 1990) were found to concentrate preening at their suture location, shortly after transmitter implantation. Similar observations were found with the living cormorants; cormorant 3 was observed preening the biopsy site on the breast within minutes of being released back into its holding pen. This bird had its wounds closed with tissue glue and no bleeding occurred during the healing process, despite preening the biopsy site. Wounds closed with sutures and subsequently preened may have allowed the wound to open slightly. This may have happened with cormorant 86. On day 2 post-sampling, the wound

seemed to have remained closed and no blood was present (Appendix F, Figure 3), whereas on day 6 post-sampling there was blood present and the wound appeared larger (Appendix F, Figure 7). Cormorant 86 may have pulled on the sutures while preening and opened up the wound.

The tissue glue was faster to apply and permitted the wound to heal faster than sutures. Sutures take longer to apply (Trott 1997), the wounds have a greater chance of developing infection (Vanholder et al. 1993), and the tools needed to apply sutures also pose a threat to the individual suturing the bird. Sharp-tipped sutures cause 51-77% of percutaneous injuries for human surgeons (Department of Health and Human Service 2007). Typically human patients remain still while they are being sutured, whereas birds are known to struggle while being handled (Maechtle 1998). This would greatly increase the chance that a biologist might get a percutaneous injury. Birds are a carrier of West Nile virus in the U. S. and within the past few years there has been a threat of a HPAI virus strain, H5N1 subtype, of avian influenza outbreak in North America (McLean 2007) and either of these diseases could infect biologists if they get a percutaneous injury.

The breast and patagium was found to heal at the same rate. With this in mind, it can be argued that the patagium is the more suitable location to take a skin sample. As mentioned previously, there are fewer blood vessels located there, decreasing the chance of an infection reaching the blood stream (Beal et al. 2000, Muza et al. 2000), and there are fewer muscles involved in flight that could be damaged from a skin sample biopsy (Proctor and Lynch 1993).

MANAGEMENT IMPLICATIONS

Now that an aging study has been completed we can critique the procedure to make it more humane for the birds and easier for the biologist. We recommend that for future studies the Ps skin samples should be taken from the patagium only since there is less of a chance of flight

muscle injury and infection. Tissue glue is quick to apply, effective, and should be used to close skin biopsy wounds in the future. To prevent infection from occurring, an antibiotic may be used to treat the wound before the bird is released. Taking that into consideration, we believe that wild birds could easily be sampled in the field and released without concern that they will develop complications post-biopsy.

This aging method has numerous applications for wildlife management. It could aid in any study where age is a factor. The age curves were found to produce age estimates to within 1½ - 2½ years of actual ages. Aside from long term banding studies, no other aging method has produced age estimates to this precision after the bird has reached adulthood. Within a matter of months an entire population could be sampled and age demographics could be calculated. This could be incredibly useful for assessing population status, diagnosing the causes of poor population performance, prescribing management tactics, and making prognoses of population viability. Knowing age demographics of bird populations can provide researchers with information to help them prevent native populations from dwindling and invasive species populations from growing at an exponential rate. For pest species, such as cormorants, and endangered and threatened species, such as California condors *(Gymnogyps californianus*), obtaining age demographics for a population could provide insight into what management practice works best for different age classes. This could not only produce healthier populations, but also create better human/wildlife interactions.

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TABLES

Table 1. Statistics for known-aged cormorant breast and patagial skins (n = 63) when estimated ages were calculated with 5 different age curves and compared to actual ages.

	Breast Skin				Patagial Skin			
Curve	*P* Value[a]	t_{62} Value	$\bar{x}$ (months)	SE	*P* Value	t_{62} Value	$\bar{x}$ (months)	SE
Fallon et al. (2006*b*)	<0.0001	6.36	37.6	5.9	<0.0001	7.33	40.9	5.6
Our linear breast skin	0.62	-0.49	3.6	7.3	0.70	0.38	2.6	6.7
Our linear patagial skin	0.22	-1.24	8.7	7.1	0.22	-1.24	2.7	6.5
Our curvilinear breast skin	0.12	1.59	14.5	9.2	0.27	1.12	9.7	8.7
Our curvilinear patagial skin	0.07	1.86	16.1	8.6	0.17	1.37	8.3	6.0

[a]*P* values were compared to an alpha value of 0.05.

Table 2. Statistics for known-aged cormorant 6 mm^2 and 20 mm^2 patagial skins (n = 50) when estimated ages were calculated with our patagial skin age curves and compared to actual ages.

	6 mm^2 Skins				20 mm^2 Skins			
Curve	*P* Value[a]	t_{49} Value	$\bar{x}$ (months)	SE	*P* Value	t_{49} Value	$\bar{x}$ (months)	SE
Our linear patagial	0.08	-1.74	11.7	6.8	0.87	0.16	1.2	7.5
Our curvilinear	0.04	-2.07	18.2	8.8	0.43	-0.80	7.8	9.8

[a]*P* values were compared to an alpha value of 0.05.

Table 3. Estimated ages (months) determined from breast and patagial skins using the Fallon et al. (2006*a*) breast skin age curve, our linear and curvilinear breast skin age curve, and our linear and curvilinear patagial skin age curve for each of the 7 living cormorants captured at Noxoubee National Wildlife Refuge, Mississippi, 2008.

Bird	Sexually Mature	Gender	Location	Pentosidine concentration (pmol Ps/mg collagen)	Estimated age (months) Fallon age curve	Estimated age (months) our breast skin age curve (linear)	Estimated age (months) our patagial skin age curve (linear)	Estimated age (months) our breast skin age curve (curvilinear)	Estimated age (months) our patagial skin age curve (curvilinear)	Actual age (months)
AJL	Yes	Female	Breast	24.93	95[a]	206	207	242	236	32
AJL			Patagium	12.43	30[b]	77	82	60	66	
3	No	Male	Breast	8.60	10	38	44	29	34	U[c]
3			Patagium	8.82	11	40	46	30	36	
18	No	Male	Breast	6.92	1	21	27	19	23	U
18			Patagium	6.89	1	20	27	19	23	
32	Yes	Male	Breast	16.43	51	118	122	105	110	U
32			Patagium	13.85	38	92	96	76	82	
34	No	Male	Breast	15.03	44	104	108	88	94	U
34			Patagium	14.10	39	95	99	78	84	
37	No	Female	Breast	8.46	9	37	43	28	33	U
37			Patagium	8.49	10	37	43	28	33	
86	Yes	Male	Breast	22.49	83	181	182	198	196	U
86			Patagium	21.12	75	167	169	174	174	

[a]Estimated ages were determined by placing the Ps concentration into the y value of the regression equations and solving for x.

[b]The largest difference in age estimates for all 5 age curves were from the breast and patagium of bird AJL.

[c]U = Unknown.

FIGURES

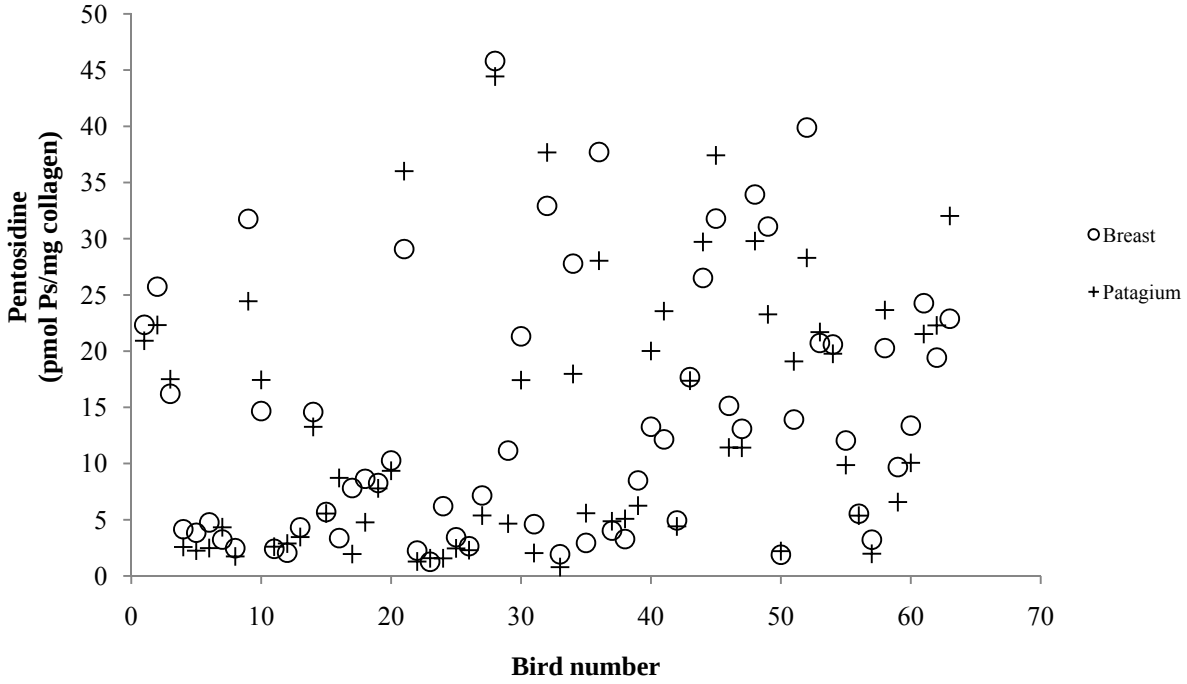

Figure 1. Distribution of pentosidine (Ps) concentrations for deceased cormorants from the breeding and wintering grounds in the U.S. in 2008. Pentosidine concentrations were higher in the breast (65% of the samples) more often than the patagium. The majority (78%) of the different body parts had Ps concentrations within 5 pmol Ps/mg collagen of each other. Only 2 birds (birds 41 and 52) had a Ps concentration difference over 10 pmol Ps/mg collagen for its body parts.

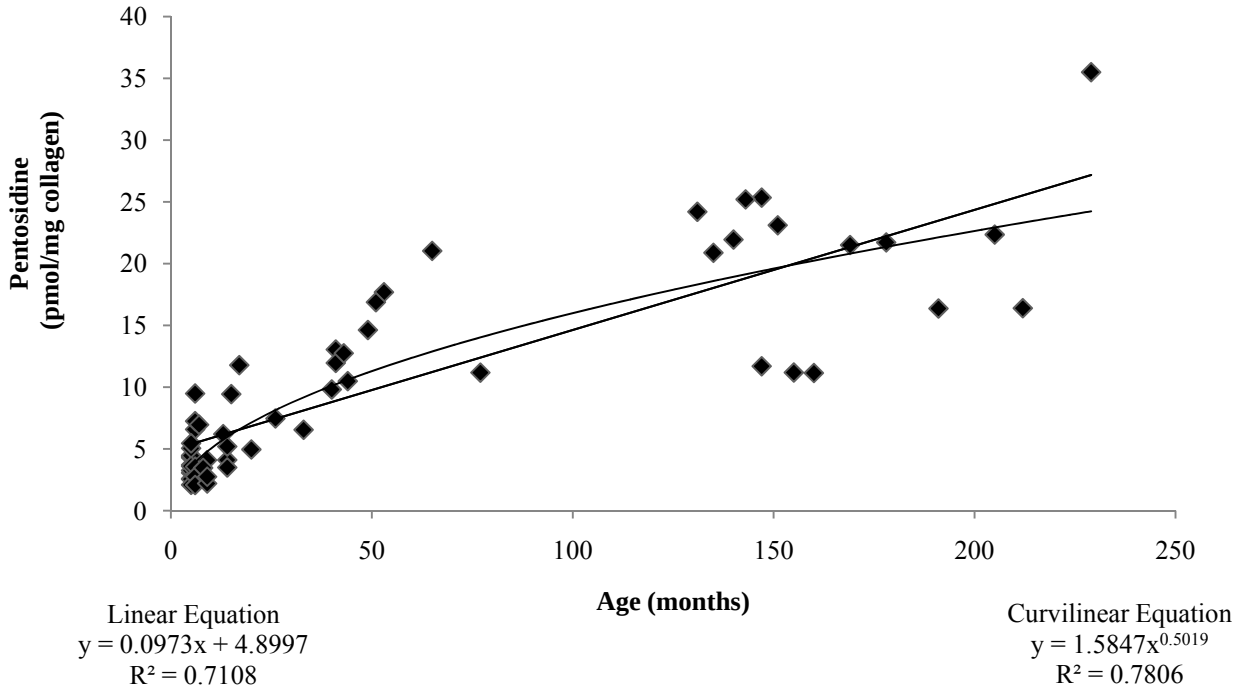

Figure 2. Cormorant age curve generated from breast data (n = 58) from deceased cormorants collected from the breeding and wintering grounds in the U.S. in 2008. Known-aged cormorants ranged from 5 to 229 months old. The youngest birds have the lowest concentration of pentosidine (Ps) and the oldest bird has the highest concentration of Ps. There is a lack of known-age birds from 77 to 131 months old. A positive linear relation is apparent. The curvilinear regression (R^2 = 0.7806) has a better fit to the data compared to the linear regression (R^2 = 0.7108).

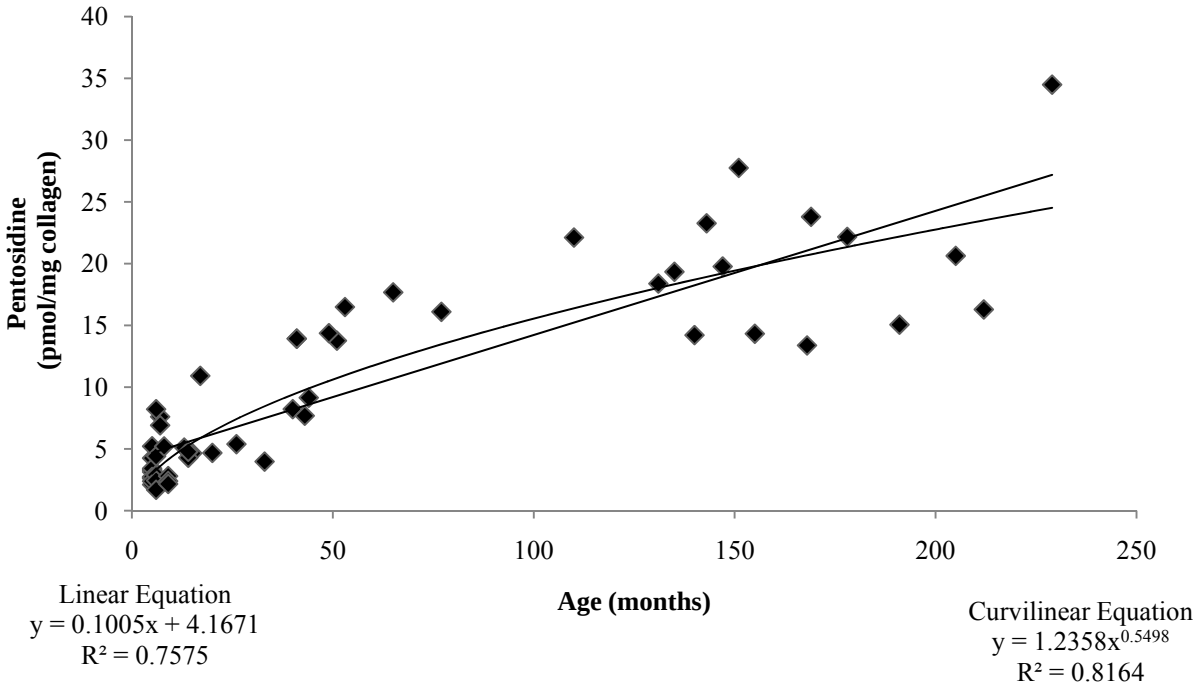

Figure 3. Cormorant age curve generated from patagial data (n = 58) from deceased cormorants collected from the Mississippi Delta region in Mississippi in 2008. Known-aged cormorants ranged from 5 to 229 months old. The youngest birds have the lowest concentration of pentosidine (Ps) and the oldest bird has the highest concentration of Ps. There is a lack of known-age birds from 77 to 110 months old. A positive linear relation is apparent. The curvilinear regression (R^2 = 0.8164) has a better fit to the data compared to the linear regression (R^2 = 0.7575).

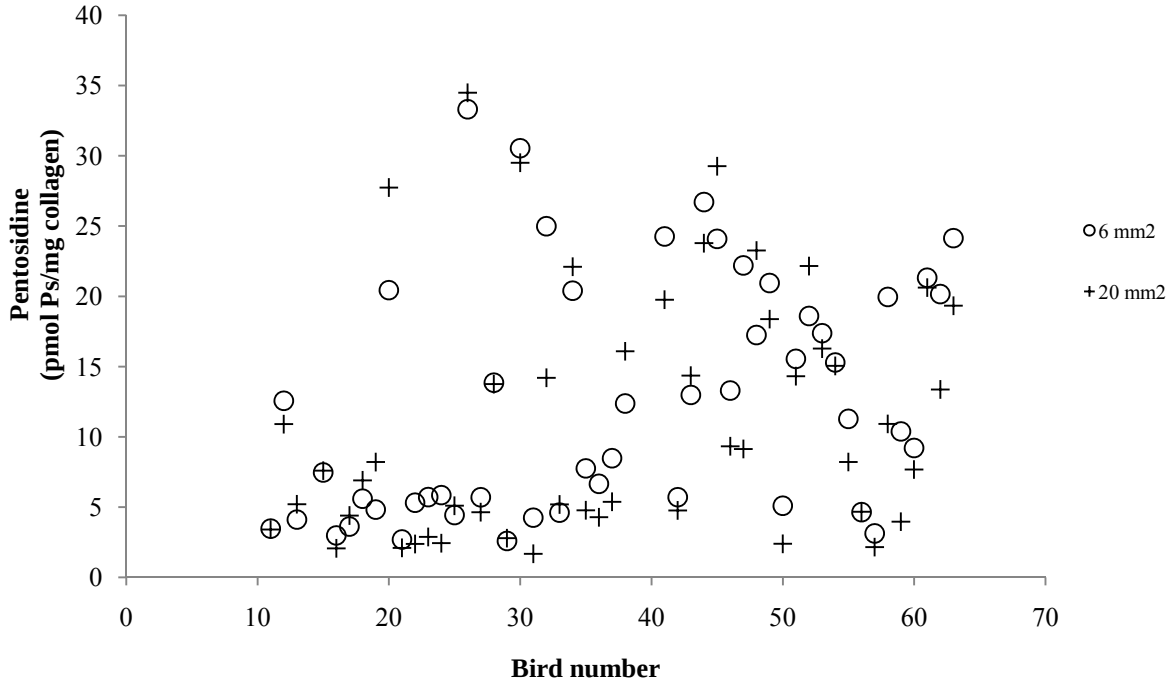

Figure 4. Pentosidine (Ps) concentration comparisons for 6 mm^2 and 20 mm^2 patagial skin samples from deceased cormorants from the Mississippi Delta region in Mississippi in 2008. Cormorants 1-10, 14, 39, and 40 were not processed for 2 different skin sizes. The majority of the birds (66%) had higher Ps concentrations in the 6 mm^2 skins. For 84% of the birds, there was less than a difference of 5 pmol Ps/mg collagen between the 2 skin sizes. Birds 32 and 47 had the highest differences of Ps concentration (10.779 pmol Ps/mg collagen and 13.057 pmol Ps/mg collagen respectively).

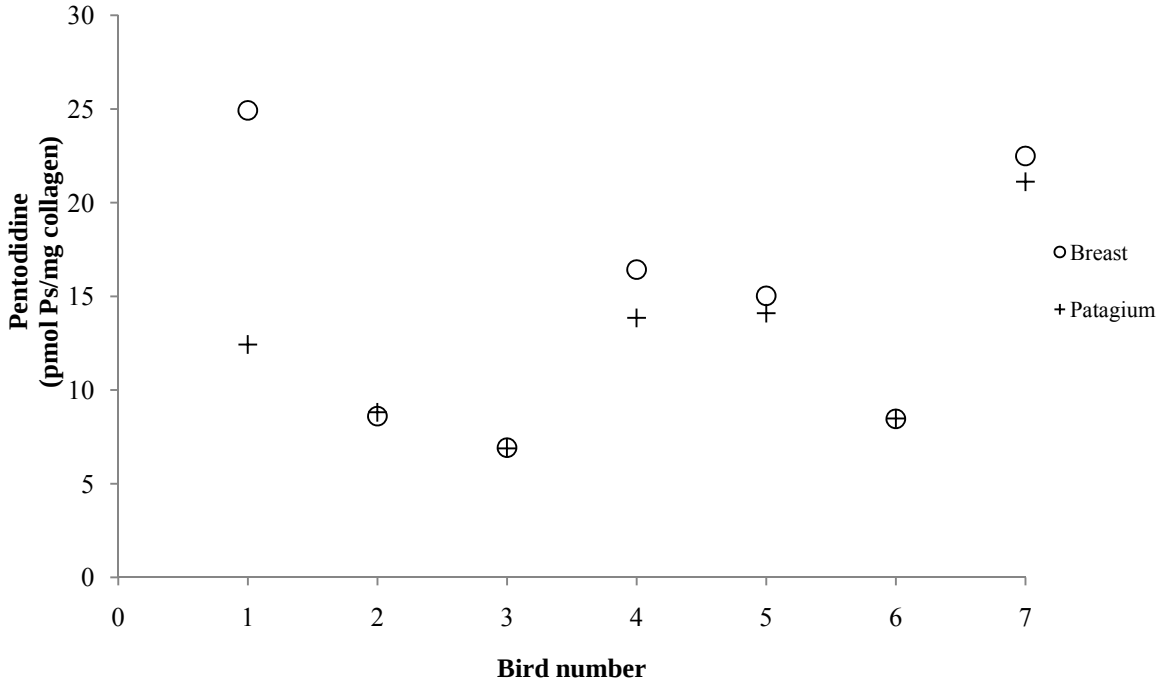

Figure 5. Distribution of pentosidine (Ps) concentrations for the breast and patagium of 7 living cormorants captured from Mathews Break National Wildlife Refuge, Mississippi, in 2008. For 71% of the birds, the Ps concentration was higher in the breast. The majority of the cormorants (86%) had a difference in Ps concentration less than 3 pmol Ps/mg collagen. Cormorant 1 had the largest difference in Ps concentrations (12.5 pmol Ps/mg collagen).

APPENDIX A. CHEMICAL SOLUTIONS FOR HYDROXYPROLINE ASSAY

Table 1. Chloramine T solution.

Chemical	25 Samples	50 Samples	100 Samples
Chloramine T buffer	0.087 g	0.175 g	0.350 g
ddi H_2O	2.5 ml	5.0 ml	10.0 ml
n-Propanol	2.5 ml	5.0 ml	10.0 ml
Buffer A	20.0 ml	40.0 ml	80.0 ml

Table 2. Color reagent solution.

Chemical	25 Samples	50 Samples	100 Samples
p-dimethylamino-benzaldehyde	3.75 g	7.5 g	15.0 g
n-Propanol	15.0 ml	30.0 ml	60.0 ml
60% Perchloric acid	6.5 ml	13.0 ml	26.0 ml
n-Propanol	3.5 ml	7.0 ml	14.0 ml

Table 3. Stock hydroxyproline buffer.

Chemical	Amount
Citric acid monohydrate-analytical grade	50 g
Acetic acid (96%)	12 ml
Sodium acetate trihydrate-analyical grade	120 g
Sodium hydroxide	34 g
ddi H_2O	to 1000 ml
Toluene	10 drops

Table 4. Buffer A solution.

Chemical	Amount
Stock hydroxyproline buffer	500 ml
ddi H_2O	100 ml
n-Propanol	150 ml

Table 5. Buffer B solution.

Chemical	Amount
Buffer A	100 ml
ddi H_2O	400 ml

APPENDIX B. EXTRA TABLE FROM THE BLACK VULTURE DATA

Table 1. Pentosidine concentrations, estimated ages (months) from the double-crested cormorant and the wild bird curve, and difference in estimated ages (months) from the 2 age curves for breast and patagial skins of 30 black vultures from Gainesville, FL, USA in 2007.

Bird number	Location	Age class	Ps concentration (pmol Ps/mg collagen)	Estimated age (months) from cormorant curve	Estimated age (months) from wild bird curve	Difference in age estimate (months)
1	Breast	Adult	6.33	-2[a]	-6	4
1	Patagium	Adult	9.74	16	11	5
2	Breast	Sub-adult	3.52	-16	-19	3
2	Patagium	Sub-adult	8.84	11	7	5
3	Breast	Adult	7.69	5	1	4
3	Patagium	Adult	11.33	24	19	6
4	Breast	Adult	5.83	-4	-8	4
4	Patagium	Adult	6.18	-3	-6	4
6[b]	Breast	Adult	8.93	12	7	5
6	Patagium	Adult	12.72	32	26	6
7	Breast	Adult	6.26	-2	-6	4
7	Patagium	Adult	6.09	-3	-7	4
8	Breast	Juvenile	10.52	20	15	5
8	Patagium	Juvenile	9.81	16	11	5
9	Breast	Adult	14.55	41	35	7
9	Patagium	Adult	10.57	20	15	5
11	Breast	Adult	10.38	19	14	5
11	Patagium	Adult	11.18	24	18	5
12	Breast	Adult	7.22	3	-1	4

12	Patagium	Adult	8.70	11	6	5
13	Breast	Juvenile	9.18	13	8	5
13	Patagium	Juvenile	5.71	-5	-9	4
14	Breast	Adult	5.93	-4	-8	4
14	Patagium	Adult	6.77	1	-3	4
15	Breast	Juvenile	8.26	8	4	4
15	Patagium	Juvenile	5.47	-6	-10	4
16	Breast	Juvenile	10.45	20	15	5
16	Patagium	Juvenile	5.30	-7	-11	3
17	Breast	Sub-Adult	11.51	25	20	6
17	Patagium	Sub-Adult	11.93	27	22	6
18	Breast	Sub-Adult	11.55	26	20	6
18	Patagium	Sub-Adult	12.92	33	27	6
19	Breast	Adult	5.53	-6	-10	4
19	Patagium	Adult	10.48	20	15	5
20	Breast	Adult	12.84	32	26	6
20	Patagium	Adult	13.41	35	29	6
21	Breast	Juvenile	6.38	-2	-5	4
21	Patagium	Juvenile	4.48	-11	-15	3
22	Breast	Adult	14.17	39	33	6
22	Patagium	Adult	8.40	9	5	5
23	Breast	Adult	6.63	0	-4	4
23	Patagium	Adult	8.68	11	6	5
24	Breast	Adult	11.15	23	18	5
24	Patagium	Adult	12.42	30	24	6
25	Breast	Adult	12.40	30	24	6
25	Patagium	Adult	10.22	19	13	5
26	Breast	Adult	6.93	1	-3	4
26	Patagium	Adult	7.11	2	-2	4

27	Breast	Adult	12.58	31	25	6
27	Patagium	Adult	8.24	8	4	4
28	Breast	Adult	8.60	10	6	5
28	Patagium	Adult	10.11	18	13	5
29	Breast	Adult	7.91	6	2	4
29	Patagium	Adult	9.07	13	8	5
30	Breast	Juvenile	6.20	-2	-6	4
30	Patagium	Juvenile	4.19	-13	-16	3

[a]Birds with negative estimated ages were aged as being <1 – 6 months old.

[b]Vultures 5 and 10 were removed from all analyses because one was an outlier and one was missing a Ps value.

APPENDIX C. EXTRA TABLE FROM THE MONK PARAKEET DATA

Table 1. Pentosidine concentrations (pmol Ps/mg collagen), actual ages (months) and estimated ages (months) for breast and patagial skins of 105 monk parakeets from Gainesville, FL, USA in 2007.

Bird	Location	Ps Concentration (Ps/mg collagen)	Actual age (months)	Estimated age (months)
A003	Breast	22.83	60+[a]	75
A003	Patagium	25.07	60+	86
A008	Breast	20.13	42+	62
A008	Patagium	24.06	42+	81
A012	Breast	37.53	54+	147
A012	Patagium	35.51	54+	137
A018	Breast	24.19	42+	82
A018	Patagium	13.63	42+	30
A026	Breast	30.13	60+	111
A026	Patagium	35.49	60+	137
A040	Breast	31.95	54+	120
A040	Patagium	18.15	54+	52
A042	Breast	32.51	54+	122
A042	Patagium	26.02	54+	91
A048	Breast	24.81	54+	85
A048	Patagium	32.48	54+	122
A050	Breast	43.78	60+	177
A050	Patagium	26.75	60+	94
A053	Breast	23.65	60+	79
A053	Patagium	32.26	60+	121
A054	Breast	59.62	60+	255
A054	Patagium	23.59	60+	79
A055	Breast	36.37	60+	141

A055	Patagium	37.85	60+	148
A057	Breast	33.98	42+	129
A057	Patagium	20.25	42+	62
A058	Breast	19.72	36+	60
A058	Patagium	42.64	36+	172
A066	Breast	24.32		82
A066	Patagium	14.15		33
A070	Breast	19.12	54+	57
A070	Patagium	18.54	54+	54
A072	Breast	19.81	54+	60
A072	Patagium	18.54	54+	54
A074	Breast	26.66	24+	94
A074	Patagium	13.82	24+	31
A082	Breast	25.12	54+	86
A082	Patagium	28.55	54+	103
A088	Breast	31.71	42+	118
A088	Patagium	16.44	42+	44
A090	Breast	45.93	54+	188
A090	Patagium	29.55	54+	108
A100	Breast	35.34	24+	136
A100	Patagium	16.17	24+	42
A113	Breast	23.37	36+	78
A113	Patagium	46.59	36+	191
A115	Breast	19.92	42+	61
A115	Patagium	25.12	42+	86
A116	Breast	22.17	42+	72
A116	Patagium	39.44	42+	156
A120	Breast	10.11	18	13
A120	Patagium	7.45	18	0

C16	Breast	45.48		186
C16	Patagium	24.19		82
C17	Breast	24.42		83
C17	Patagium	23.46		78
C18	Breast	22.56		74
C18	Patagium	16.08		42
C19	Breast	6.50		-5
C19	Patagium	1.39		-30
C20	Breast	5.42		-10
C20	Patagium	2.35		-25
C21	Breast	5.19		-11
C21	Patagium	5.61		-9
C22	Breast	49.20		204
C22	Patagium	32.11		120
C23	Breast	46.57		191
C23	Patagium	35.45		137
C24	Breast	10.91		17
C24	Patagium	5.40		-10
C25	Breast	3.49		-19
C25	Patagium	2.83		-23
C26	Breast	7.53		0
C26	Patagium	4.55		-14
C27	Breast	20.02		61
C27	Patagium	4.37		-15
C29	Breast	17.26		48
C29	Patagium	11.76		21
C30	Breast	20.38		63
C30	Patagium	8.65		6
C31	Breast	6.81	1	-3

C31	Patagium	2.59	1	-24
C32	Breast	6.84	1	-3
C32	Patagium	3.89	1	-18
C33	Breast	11.21	1	18
C33	Patagium	5.66	1	-9
C34	Breast	5.12	1	-12
C34	Patagium	4.35	1	-15
C92	Breast	22.57		74
C92	Patagium	15.94		41
C93	Breast	10.40		14
C93	Patagium	6.75		-4
C94	Breast	4.72		-13
C94	Patagium	2.12		-26
C95	Breast	12.03		22
C95	Patagium	8.98		7
C96	Breast	9.01		8
C96	Patagium	13.43		29
C97	Breast	4.22		-16
C97	Patagium	2.99		-22
C98	Breast	13.73		31
C98	Patagium	6.45		-5
C99	Breast	5.44		-10
C99	Patagium	3.38		-20
D1	Breast	17.14		47
D1	Patagium	6.95		-3
D2	Breast	9.84		12
D2	Patagium	5.50		-10
D25	Breast	5.04		-12
D25	Patagium	1.50		-29

D26	Breast	3.70	-18
D26	Patagium	2.03	-27
D27	Breast	10.61	15
D27	Patagium	4.73	-13
D28	Breast	31.21	116
D28	Patagium	12.26	23
D3	Breast	3.23	-21
D3	Patagium	2.08	-26
D35	Breast	3.55	-19
D35	Patagium	1.27	-30
D36	Breast	2.77	-23
D36	Patagium	2.08	-26
D37	Breast	39.46	156
D37	Patagium	17.93	51
D38	Breast	16.47	44
D38	Patagium	9.90	12
D39	Breast	5.50	-10
D39	Patagium	1.62	-29
D4	Breast	7.52	0
D4	Patagium	5.63	-9
D61	Breast	23.18	77
D61	Patagium	11.35	19
D62	Breast	41.60	167
D62	Patagium	13.76	31
D63	Breast	4.15	-16
D63	Patagium	1.68	-28
D64	Breast	2.57	-24
D64	Patagium	2.16	-26
D65	Breast	4.10	-16

D65	Patagium	1.59	-29
D66	Breast	4.62	-14
D66	Patagium	1.62	-29
D67	Breast	5.76	-8
D67	Patagium	1.79	-28
D68	Breast	3.23	-21
D68	Patagium	1.51	-29
D69	Breast	4.76	-13
D69	Patagium	1.73	-28
D70	Breast	21.25	67
D70	Patagium	11.36	19
D71	Breast	23.78	80
D71	Patagium	11.27	19
D72	Breast	24.02	81
D72	Patagium	10.25	14
D73	Breast	4.44	-15
D73	Patagium	3.49	-19
D74	Breast	8.73	6
D74	Patagium	2.95	-22
D75	Breast	3.81	-18
D75	Patagium	3.12	-21
D76	Breast	5.42	-10
D76	Patagium	3.61	-19
D77	Breast	19.71	60
D77	Patagium	8.93	7
D78	Breast	4.74	-13
D78	Patagium	16.36	43
D79	Breast	4.72	-13
D79	Patagium	2.24	-26

D80	Breast	4.64		-14
D80	Patagium	1.83		-28
D81	Breast	4.59		-14
D81	Patagium	3.42		-20
D82	Breast	4.28		-16
D82	Patagium	1.78		-28
D83	Breast	5.16		-11
D83	Patagium	3.70		-18
D84	Breast	27.22		96
D84	Patagium	15.44		39
D85	Breast	30.87		114
D85	Patagium	17.67		50
D86	Breast	4.32		-15
D86	Patagium	2.59		-24
D87	Breast	4.10		-16
D87	Patagium	2.06		-26
D88	Breast	10.89		17
D88	Patagium	2.25		-26
H15	Breast	6.15	2	-6[b]
H15	Patagium	10.06	2	13
H16	Breast	4.99	2	-12
H16	Patagium	8.29	2	4
H17	Breast	8.72	2	6
H17	Patagium	4.15	2	-16
H18	Breast	8.84	2	7
H18	Patagium	7.62	2	1
H26	Breast	2.36	4.5	-25
H26	Patagium	5.76	4.5	-8
H30	Breast	4.89	4	-13

H30	Patagium	4.10	4	-16
H31	Breast	2.72	4	-23
H31	Patagium	1.41	4	-30
H33	Breast	4.35	5	-15
H33	Patagium	2.53	5	-24
H34	Breast	2.96	5	-22
H34	Patagium	1.49	5	-29
H35	Breast	3.13	5	-21
H35	Patagium	0.97	5	-32
H36	Breast	3.25	5	-21
H36	Patagium	4.64	5	-14
H38	Breast	4.07	5	-17
H38	Patagium	0.91	5	-32

[a]Birds that had a known-age of (#)+ were a minimum of that many months old.

[b]Birds with negative estimated ages were aged as being <1 – 6 months.

APPENDIX D. ESTIMATED AGES FOR THE DECEASED CORMORANTS USING BREAST AND PATAGIAL SKINS

Table 1. Comparison of estimated ages (months) determined from breast and patagial skins using the Fallon et al. (2006*a*) age curve, our linear and curvilinear breast skin age curves, and our linear and curvilnear patagial skin age curves to real ages determined by banding records for cormorants collected from the breeding and wintering grounds in the U.S. in 2008.

Bird number	Location	Estimated age (months) Fallon et al. age curve	Estimated age (months) our breast skin age curve (linear)	Estimated age (months) our patagial skin age curve (linear)	Estimated age (months) our breast skin age curve (curvilinear)	Estimated age (months) our patagial skin age curve (curvilinear)	Actual age (months)
1	Breast	**58**[a]	131	134	122	126	53
1	Patagium	**51**	119	123	106	111	53
2	Breast	**75**	166	168	173	173	65
2	Patagium	**57**	131	134	122	126	65
3	Breast	**33**	84	88	67	73	41
3	Patagium	**38**	93	97	76	82	41
4	Breast	-12[b]	-6	1	**7**	10	5
4	Patagium	-18	-16	-9	**5**	6	5
5	Breast	-16	-12	-5	**6**	7	5
5	Patagium	-21	-23	-15	3	**4**	5
6	Breast	-19	-19	-11	4	**5**	5
6	Patagium	-13	-7	1	**7**	10	5
7	Breast	**96**	206	207	243	237	41
7	Patagium	**65**	146	148	142	145	41
8	Breast	**28**	72	77	56	62	41
8	Patagium	**38**	93	97	76	82	41
9	Breast	-21	-24	-16	3	**4**	5
9	Patagium	-21	-23	-15	3	**4**	5
10	Breast	-22	-24	-16	3	**4**	5

10	Patagium	-18	-18	-10	**4**[c]	**6**	5
11	Breast	-12	-5	**3**	8	11	5
11	Patagium	-17	-15	-8	**5**	7	5
12	Breast	**27**	71	76	54	60	17
12	Patagium	**22**	62	67	47	53	17
13	Breast	-8	**2**	9	10	13	5
13	Patagium	-8	**3**	10	11	14	5
14	Breast	-12	-5	**2**	**8**	10	5
14	Patagium	-21	-22	-14	3	**4**	5
15	Breast	-17	-15	-7	5	**7**	7
15	Patagium	**5**	28	34	23	27	7
16	Breast	**-1**	17	24	17	21	6
16	Patagium	-24	-29	-21	2	**3**	6
17	Breast	**3**	24	31	21	25	6
17	Patagium	-12	-5	2	**8**	10	6
18	Breast	**1**	21	28	19	23	7
18	Patagium	**1**	21	27	19	23	7
19	Breast	**15**	47	53	35	41	6
19	Patagium	**8**	34	40	27	31	6
20	Breast	86	**187**	188	208	205	151
20	Patagium	**110**	235	235	300	287	151
21	Breast	-24	-29	-21	2	**3**	5
21	Patagium	-24	-29	-21	2	**3**	5
22	Breast	-6	**6**	13	12	15	5
22	Patagium	-22	-26	-18	2	**3**	5
23	Breast	-16	-14	-6	**5**	**7**	6
23	Patagium	-20	-21	-13	3	**5**	6
24	Breast	-20	-22	-14	3	**4**	6
24	Patagium	-22	-25	-17	2	**4**	6
25	Breast	-3	**13**	20	15	19	13

25	Patagium	-8	2	9	11	**13**	13
26	Breast	**151**	314	312	490	449	229
26	Patagium	145	304	**302**	463	426	229
27	Breast	**14**	47	52	35	40	15
27	Patagium	-11	-3	5	9	**11**	15
28	Breast	**53**	123	126	111	116	51
28	Patagium	**37**	91	95	74	80	51
29	Breast	-14	-8	-1	7	**9**	9
29	Patagium	-20	-22	-14	3	**4**	9
30	Breast	**100**	215	215	240	251	65
30	Patagium	**119**	253	252	339	321	65
31	Breast	-24	-29	-21	2	**3**	6
31	Patagium	-26	-33	-25	1	**2**	6
32	Breast	80	**175**	177	188	187	140
32	Patagium	39	96	**100**	79	85	140
33	Breast	-17	-15	-7	5	**7**	8
33	Patagium	-8	3	**10**	11	14	8
34	Breast	**120**	254	253	341	322	110
34	Patagium	**81**	177	179	191	190	110
35	Breast	-13	-8	-1	7	**9**	14
35	Patagium	-10	-1	6	9	**12**	14
36	Breast	-17	-14	-7	5	**7**	14
36	Patagium	-12	-6	1	7	**10**	14
37	Breast	4	**26**	33	22	**26**	26
37	Patagium	-7	5	12	12	**15**	26
38	Breast	24	64	**70**	49	55	77
38	Patagium	**49**	115	119	101	107	77
39	Breast	-18	-17	-9	**4**	**6**	5
39	Patagium	-18	-17	-9	**4**	**6**	5
40	Breast	-16	-13	-6	**5**	7	5

40	Patagium	-21	-22	-14	3	**4**	5
41	Breast	26	70	**75**	54	59	147
41	Patagium	68	**153**	155	**153**	155	147
42	Breast	-8	3	10	11	**14**	14
42	Patagium	-10	-1	6	9	**12**	14
43	Breast	**42**	100	104	84	90	49
43	Patagium	**40**	97	101	81	87	49
44	Breast	77	**171**	172	180	180	169
44	Patagium	89	**194**	195	221	217	169
45	Breast	**98**	210	211	250	243	147
45	Patagium	**118**	250	250	333	316	147
46	Breast	23	64	**69**	49	55	160
46	Patagium	14	46	**51**	34	40	160
47	Breast	20	57	63	**43**	49	44
47	Patagium	13	**44**	49	33	38	44
48	Breast	**97**	209	209	248	241	143
48	Patagium	87	**189**	190	211	208	143
49	Breast	**92**	198	199	228	223	131
49	Patagium	61	139	141	**132**	136	131
50	Breast	-23	-28	-20	2	**3**	9
50	Patagium	-22	-26	-18	2	**3**	9
51	Breast	24	65	**70**	49	55	155
51	Patagium	40	97	**101**	80	86	155
52	Breast	79	173	**175**	184	184	178
52	Patagium	81	**177**	**179**	192	191	178
53	Breast	51	118	**122**	105	110	212
53	Patagium	50	117	**121**	104	109	212
54	Breast	51	118	**121**	105	110	191
54	Patagium	44	104	**108**	89	94	191
55	Breast	16	50	56	**38**	43	40

55	Patagium	8	34	**40**	27	31	40
56	Breast	-9	1	8	10	**13**	20
56	Patagium	-10	-2	5	9	**11**	20
57	Breast	-21	-22	-14	3	**4**	9
57	Patagium	-24	-28	-20	2	**3**	9
58	Breast	21	60	**65**	45	51	179
58	Patagium	22	62	**67**	47	53	179
59	Breast	-1	17	**24**	17	21	33
59	Patagium	-14	-10	-2	6	**8**	33
60	Breast	**32**	81	85	64	70	43
60	Patagium	5	29	**35**	23	28	43
61	Breast	82	179	181	**195**	193	205
61	Patagium	73	162	164	166	**167**	205
62	Breast	18	53	**59**	40	45	168
62	Patagium	35	87	**92**	70	76	168
63	Breast	74	**164**	166	170	171	135
63	Patagium	66	148	151	**146**	149	135

[a]Bold text represent estimated ages closest to the real age.

[b]The negative ages are representative of birds that are less than 6 months old.

[c]Some birds had estimated ages that were tied as the closest to the actual age.

APPENDIX E. ESTIMATED AGES FOR THE DECEASED CORMORANTS USING 6mm^2 and 20mm^2 SKIN SAMPLES

Table 1. Estimated and actual ages (months) determined from 6 mm^2 and 20 mm^2 skin samples using our linear and curvilinear patagial skin age curves and banding records for cormorants collected from the breeding and wintering grounds in the U.S. in 2008.

Bird number	Skin size	Estimated age (months) our patagial skin age curve (linear)	Estimated age (months) our patagial skin age curve (curvilinear)	Actual age (months)
11	6mm^2	-7[a]	**7**[b]	5
11	20mm^2	-7	**7**	5
12	6mm^2	84	**68**	17
12	20mm^2	84	**53**	17
13	6mm^2	-1	**9**	5
13	20mm^2	**-1**	14	5
15	6mm^2	33	**26**	7
15	20mm^2	33	**27**	7
16	6mm^2	-12	**5**	6
16	20mm^2	-12	**3**	6
17	6mm^2	-5	**7**	6
17	20mm^2	-5	**10**	6
18	6mm^2	**14**	16	7
18	20mm^2	**14**	23	7
19	6mm^2	**7**	12	6
19	20mm^2	**7**	32	6
20	6mm^2	**162**	164	151
20	20mm^2	**162**	287	151

21	6mm^2	-15	**4**	5
21	20mm^2	-15	**3**	5
22	6mm^2	**11**	14	5
22	20mm^2	11	**3**	5
23	6mm^2	**15**	16	6
23	20mm^2	15	**5**	6
24	6mm^2	**17**	**17**	6
24	20mm^2	17	**4**	6
25	6mm^2	3	**10**	13
25	20mm^2	3	**13**	13
26	6mm^2	**290**	400	229
26	20mm^2	**290**	426	229
27	6mm^2	**15**	16	15
27	20mm^2	**15**	11	15
28	6mm^2	96	**81**	51
28	20mm^2	96	**80**	51
29	6mm^2	-16	**4**	9
29	20mm^2	-16	**5**	9
30	6mm^2	**262**	341	65
30	20mm^2	**262**	321	65
31	6mm^2	1	**10**	6
31	20mm^2	1	**2**	6
32	6mm^2	**207**	237	140
32	20mm^2	207	**85**	140
33	6mm^2	4	**11**	8
33	20mm^2	**4**	14	8

34	6mm^2	**162**	164	110
34	20mm^2	**162**	190	110
35	6mm^2	36	**28**	14
35	20mm^2	36	**12**	14
36	6mm^2	25	**22**	14
36	20mm^2	25	**10**	14
37	6mm^2	43	**33**	26
37	20mm^2	43	**15**	26
38	6mm^2	**82**	66	77
38	20mm^2	**82**	107	77
41	6mm^2	**200**	225	147
41	20mm^2	200	**155**	147
42	6mm^2	**15**	16	14
42	20mm^2	**15**	12	14
43	6mm^2	88	**72**	49
43	20mm^2	88	**87**	49
44	6mm^2	**224**	268	169
44	20mm^2	224	**217**	169
45	6mm^2	**198**	222	147
45	20mm^2	**198**	316	147
46	6mm^2	**91**	75	160
46	20mm^2	**91**	40	160
47	6mm^2	**179**	191	44
47	20mm^2	179	**38**	44
48	6mm^2	**130**	121	143
48	20mm^2	**130**	208	143

49	6mm^2	**167**	172	131
49	20mm^2	167	**136**	131
50	6mm^2	**9**	13	9
50	20mm^2	**9**	4	9
51	6mm^2	**113**	100	155
51	20mm^2	**113**	86	155
52	6mm^2	**144**	139	178
52	20mm^2	**144**	191	178
53	6mm^2	**131**	122	212
53	20mm^2	**131**	109	212
54	6mm^2	**111**	97	191
54	20mm^2	**111**	94	191
55	6mm^2	71	**56**	40
55	20mm^2	71	**31**	40
56	6mm^2	5	**11**	20
56	20mm^2	5	**11**	20
57	6mm^2	-10	**6**	9
57	20mm^2	-10	**3**	9
58	6mm^2	157	**158**	179
58	20mm^2	**157**	53	179
59	6mm^2	62	**48**	33
59	20mm^2	62	**8**	33
60	6mm^2	50	**39**	43
60	20mm^2	**50**	28	43
61	6mm^2	171	**178**	205
61	20mm^2	**171**	167	205

62	$6mm^2$	159	**160**	168
62	$20mm^2$	**159**	76	168
63	$6mm^2$	1**99**	223	135
63	$20mm^2$	199	**149**	135

[a]The negative ages are representative of birds that are less than 6 months old.

[b]Bold text represent estimated ages closest to the real age.

APPENDIX F. PHOTOGRAPHS OF SKIN BIOPSY WOUNDS CLOSED WITH TISSUE GLUE AND SUTURES FROM 2 CORMORANTS AS THEY HEALED

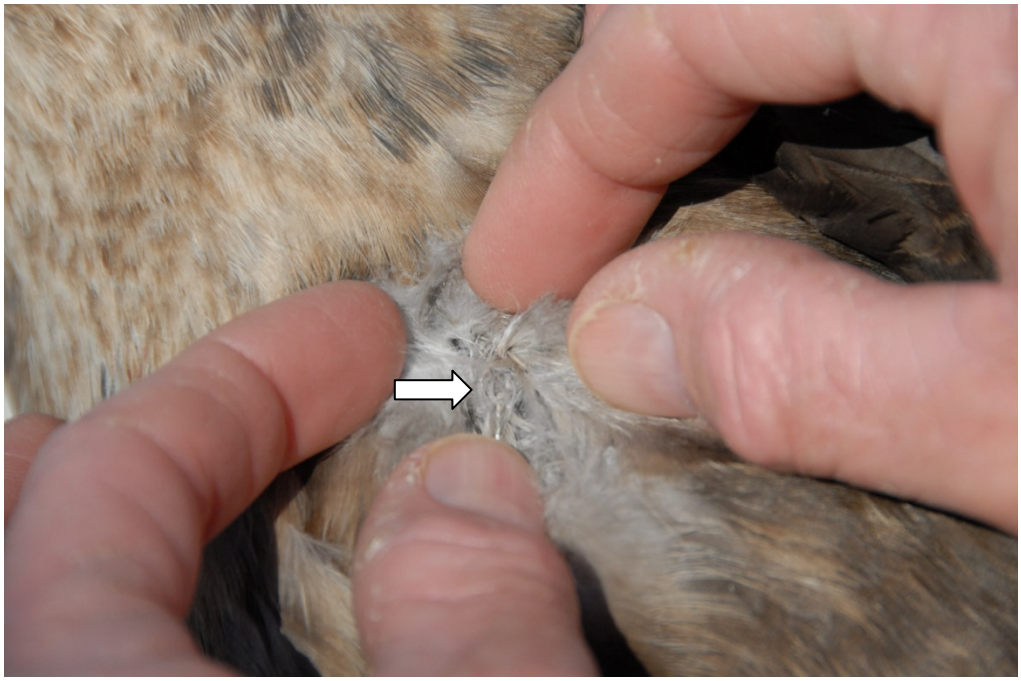

Figure 1: Photograph of cormorant 3's breast wound closed with tissue glue on 7 February 2008 (day 2 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. The wound is hidden by a mass of glue and feathers, as pointed out with the arrow. The breast shows no sign of residual bleeding.

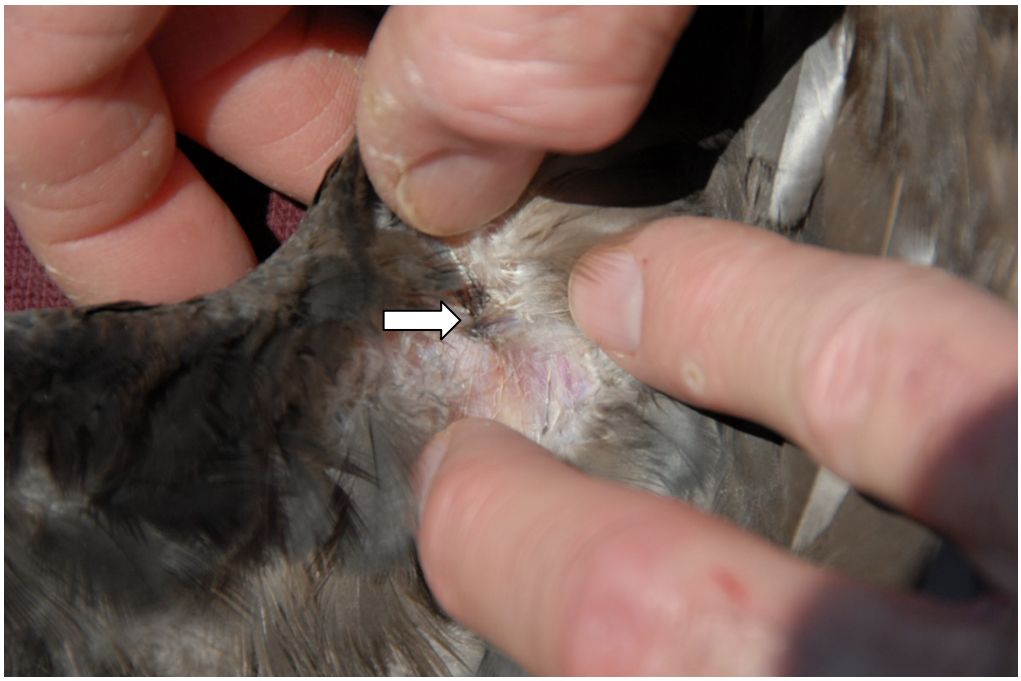

Figure 2: Photograph of cormorant 3's patagial wound closed with tissue glue on 7 July 2008 (day 2 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. The wound is still covered with glue but can be seen easily. There is no indication that the wound opened up and began bleeding again.

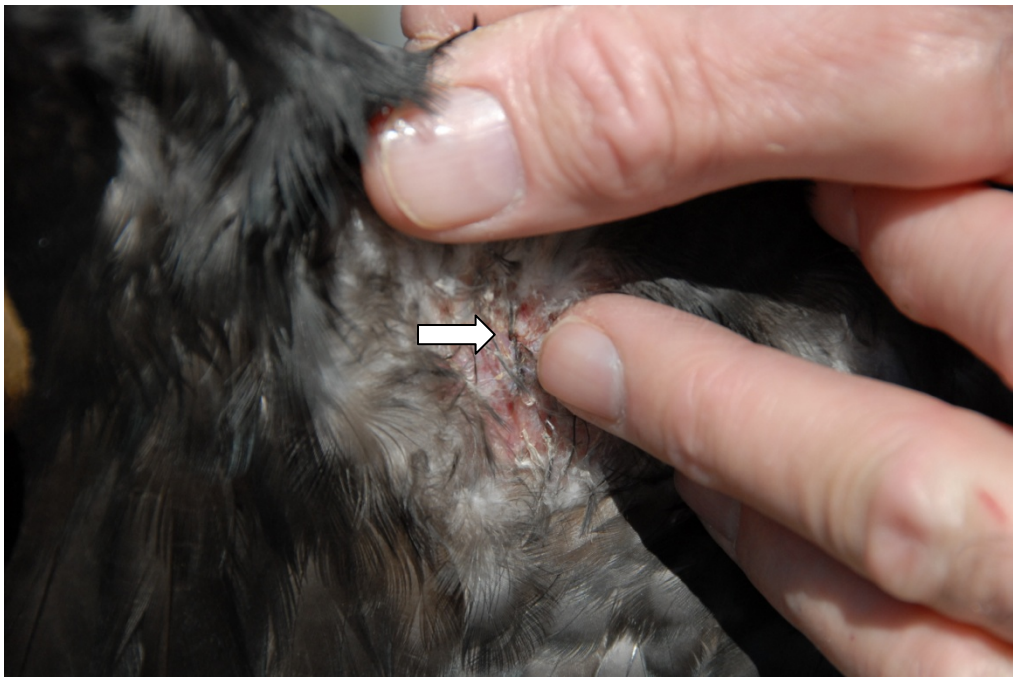

Figure 3: Photograph of cormorant 86's breast wound closed with sutures on 7 February 2008 (day 2 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. The wound remains to be held shut with the sutures. The wound is difficult to see (dark spot pointed out by arrow). There is no indication that the wound started bleeding again.

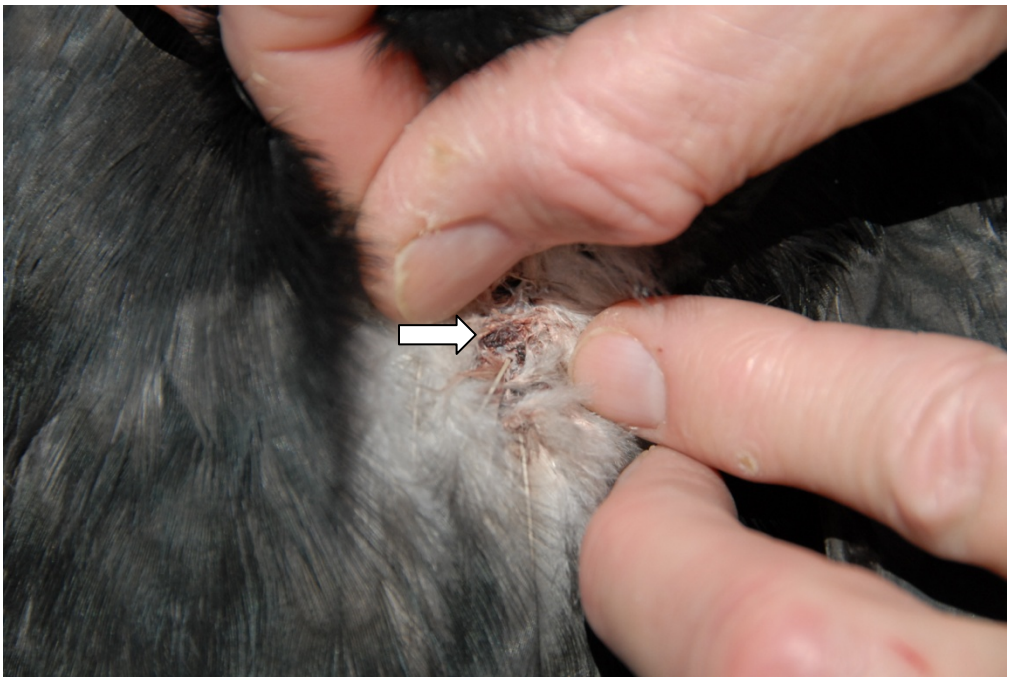

Figure 4: Photograph of cormorant 86's patagial wound closed with sutures on 7 February 2008 (day 2 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. There is a large scab that formed on the wound. There is some blood on the base of the feathers surrounding the wound, indicating that the wound opened back up.

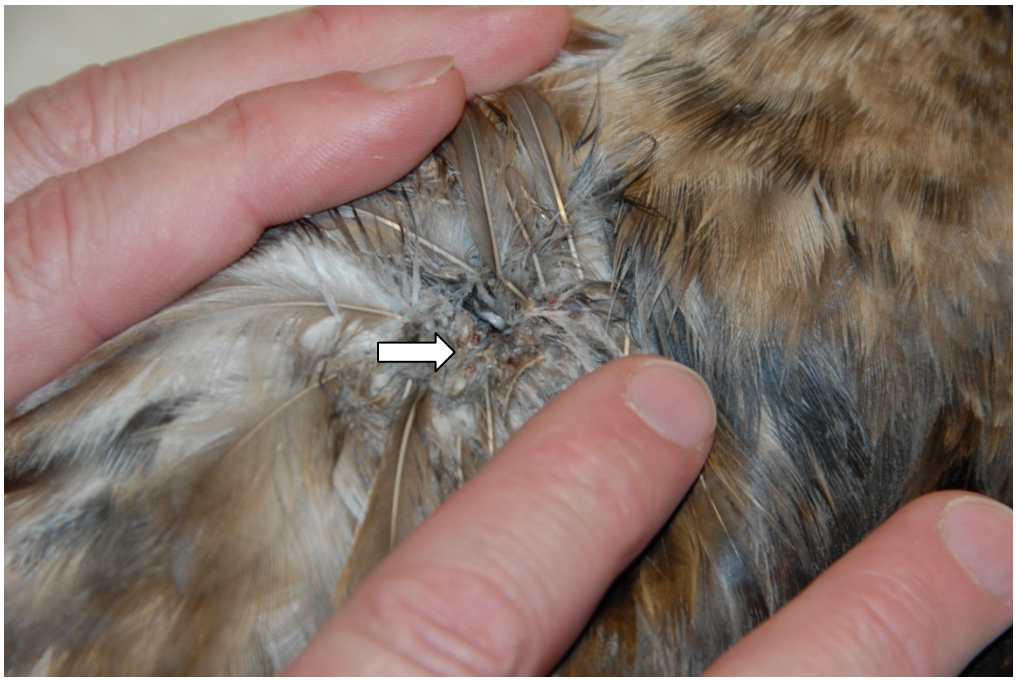

Figure 5: Photograph of cormorant 3's breast wound closed with tissue glue on 11 February 2008 (day 6 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. The glue is still holding the wound shut and it seems to be healing well.

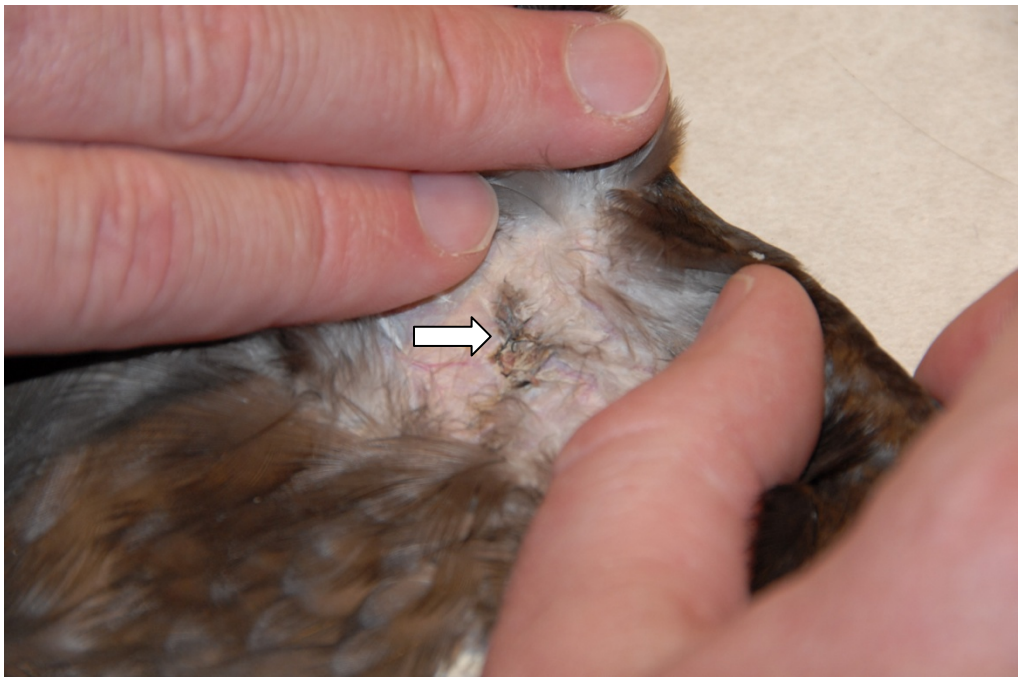

Figure 6: Photograph of cormorant 3's patagial wound closed with tissue glue on 11 February 2008 (day 6 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. The wound is still held shut by the tissue glue. It doesn't appear that the wound has healed much in 4 days.

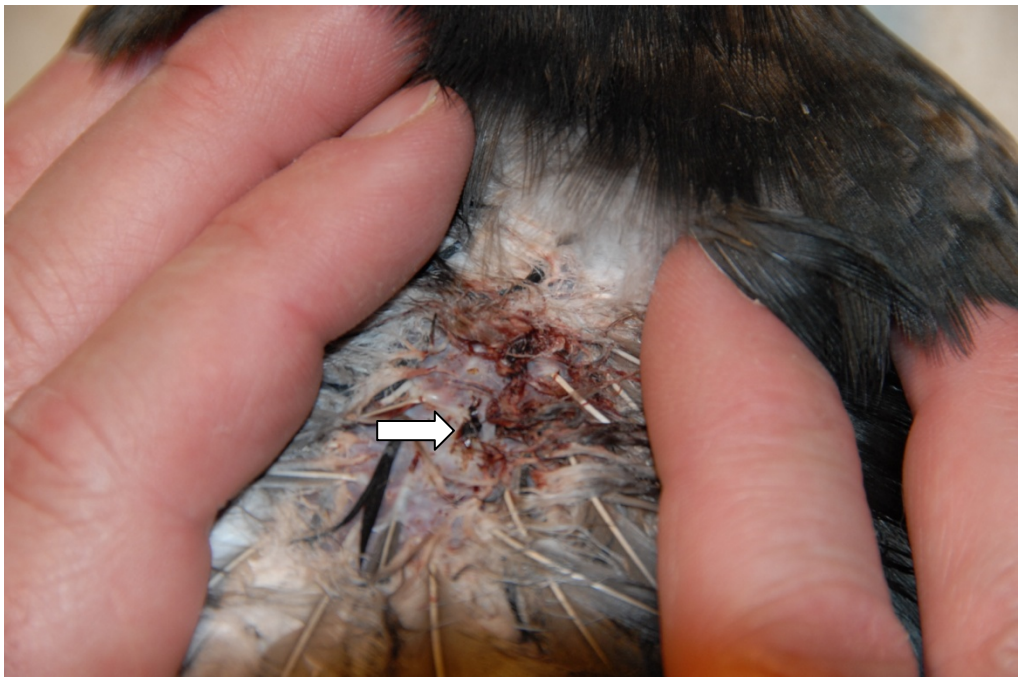

Figure 7: Photograph of cormorant 86's breast wound closed with sutures on 11 February 2008 (day 6 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. There is a lot of blood present, indicating that the wound has opened back up. The wound is really noticeable now.

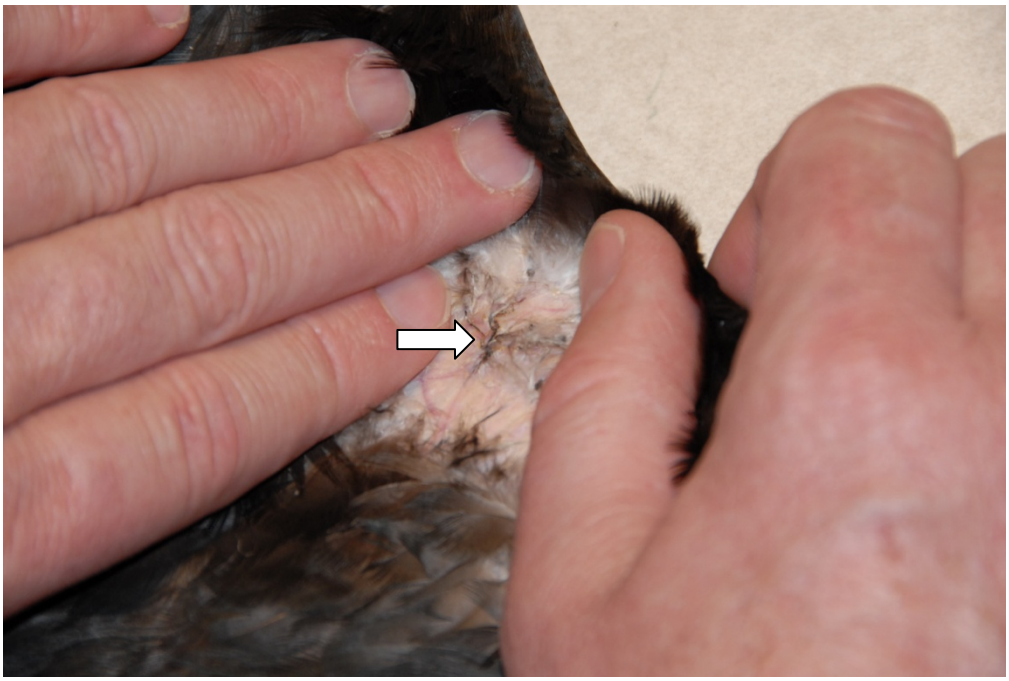

Figure 8: Photograph of cormorant 86's patagial wound closed with sutures on 11 February 2008 (day 6 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. There is no sign of bleeding, suggesting that the wound has finally closed and began healing. It appears that a feather has been caught up in the suture.

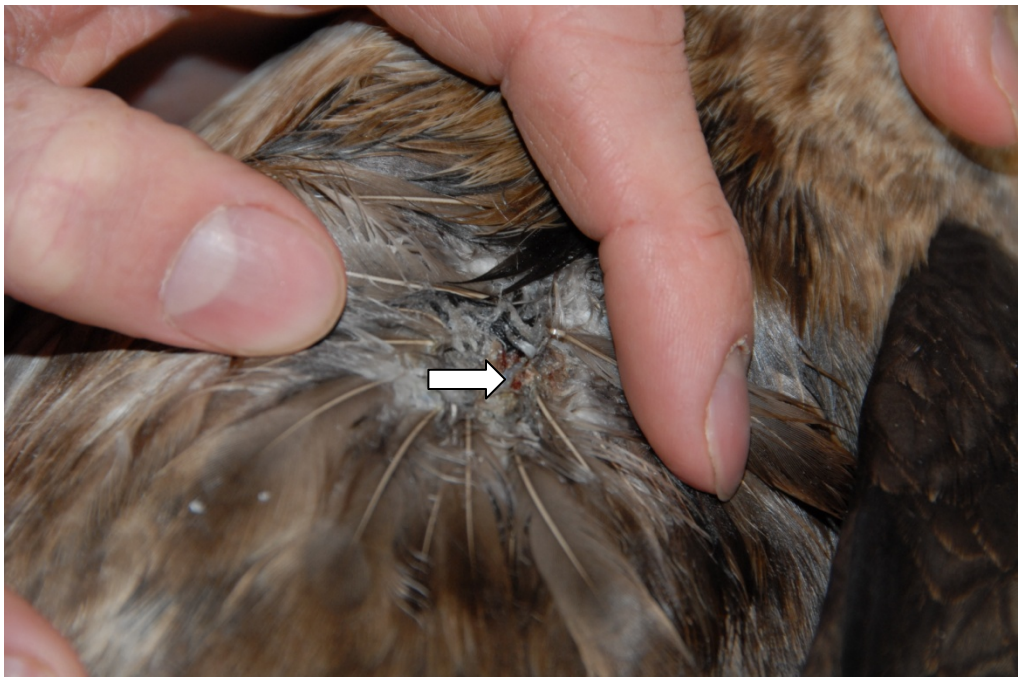

Figure 9: Photograph of cormorant 3's breast wound closed with tissue glue on 14 February 2008 (day 9 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. The wound is still healing well. The glue has not worn off.

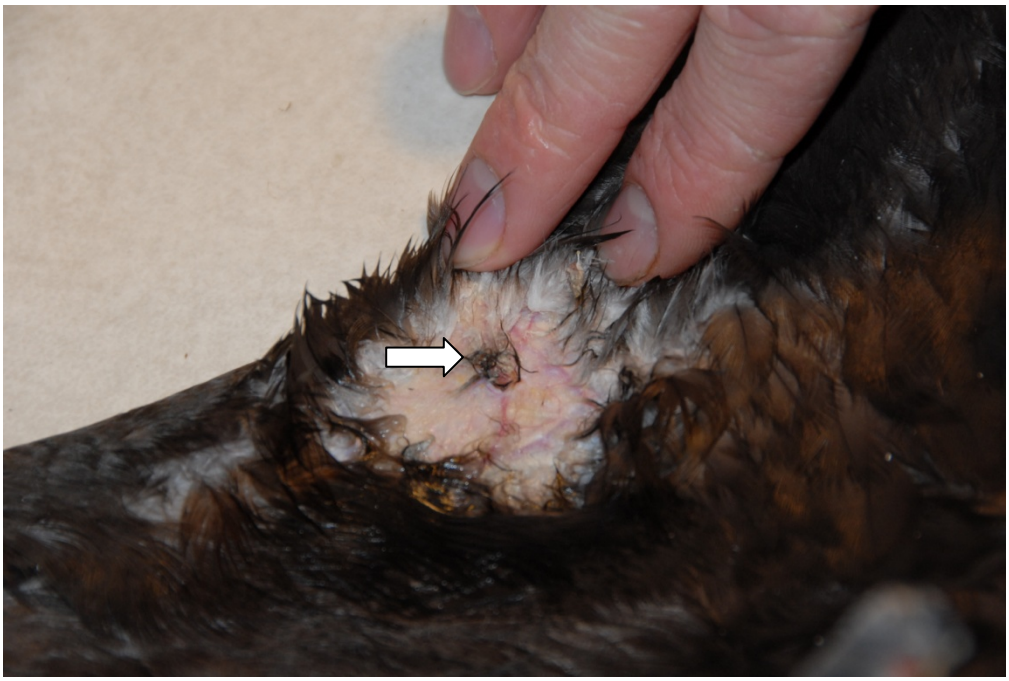

Figure 10: Photograph of cormorant 3's patagial wound closed with tissue glue on 14 February 2008 (day 9 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. There has been much improvement in healing over the last 3 days. A lot of the glue has worn away.

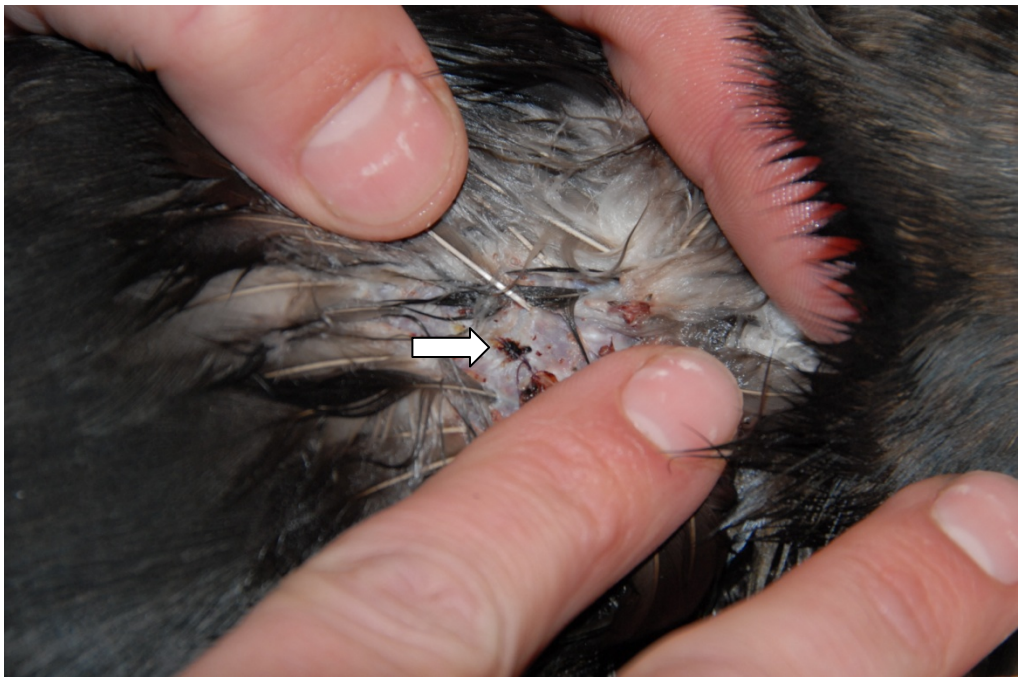

Figure 11: Photograph of cormorant 86's breast wound closed with sutures on 14 February 2008 (day 9 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. There is still some blood present around the wound, but it appears to be slowly healing.

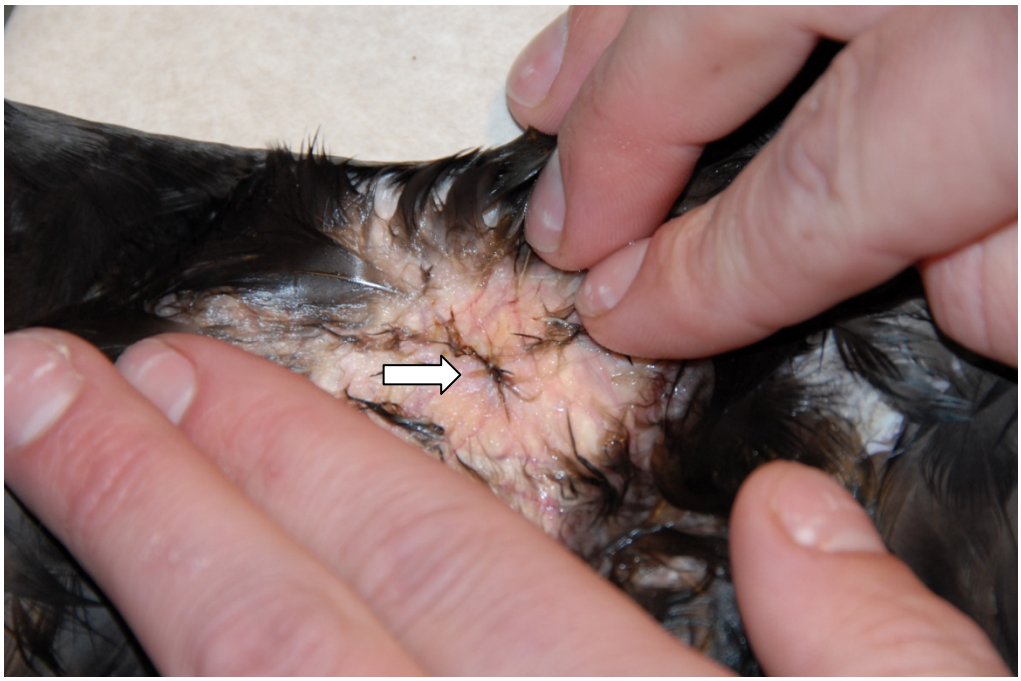

Figure 12: Photograph of cormorant 86's patagial wound closed with sutures on 14 February 2008 (day 9 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. The sutures are still holding strong and the wound is healing well. It is noticeable that a feather has been caught up in the sutures.

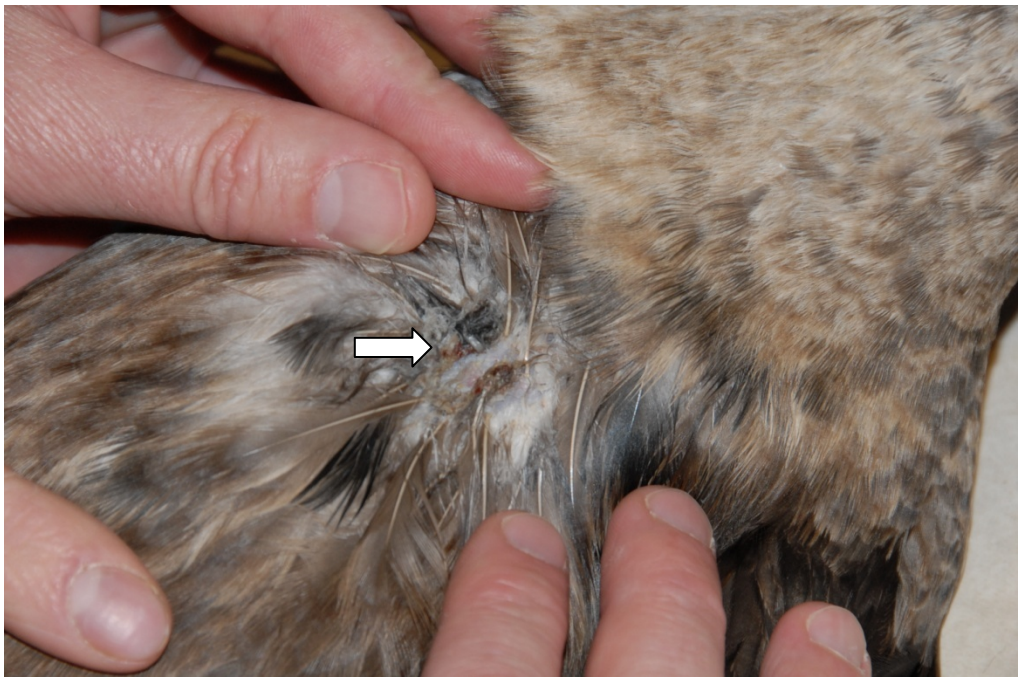

Figure 13: Photograph of cormorant 3's breast wound closed with tissue glue on 19 February 2008 (day 14 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. There is still some glue on the breast. The wound is almost completely healed.

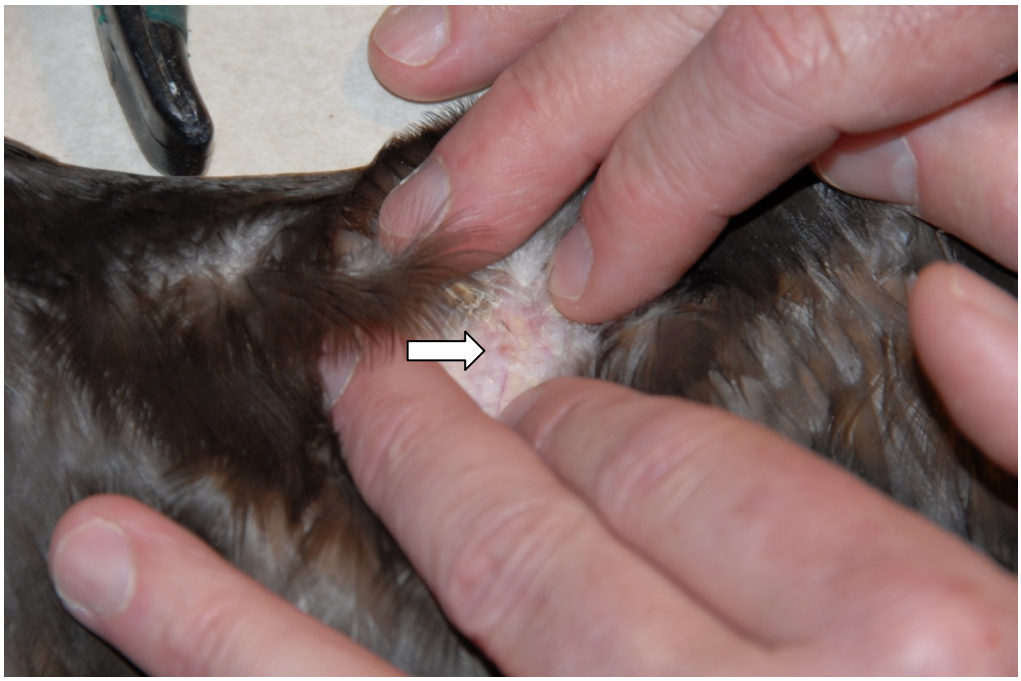

Figure 14: Photograph of cormorant 3's patagial wound closed with tissue glue on 19 February 2008 (day 14 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. There is a small red spot in the area where the biopsy was taken but the skin has completely healed. There is a little bit of glue remaining near the biopsy site (just above the arrow).

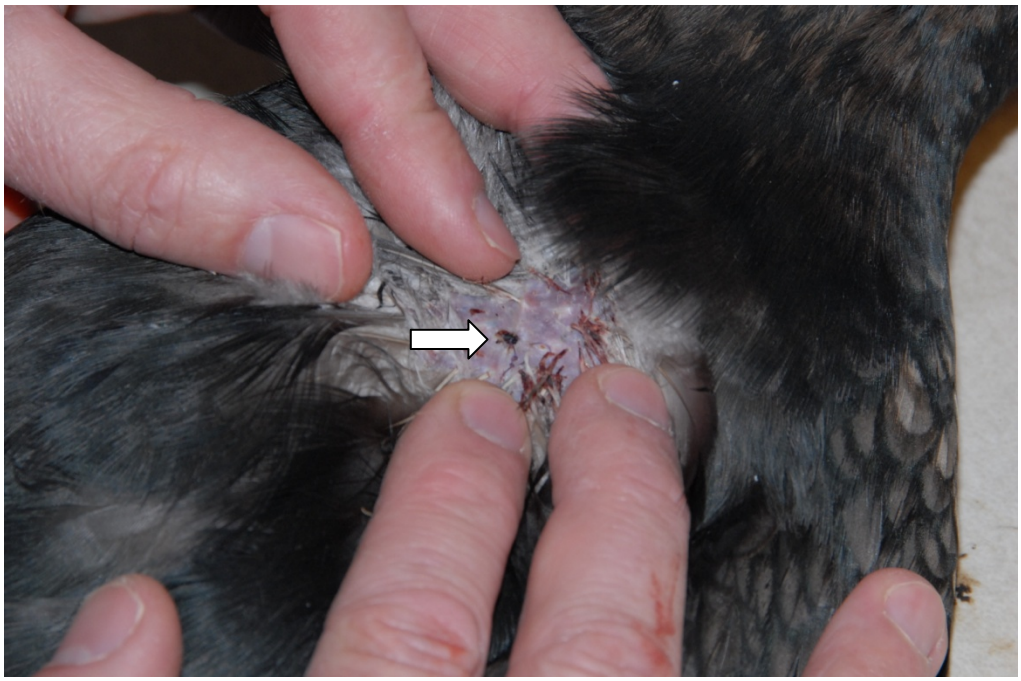

Figure 15: Photograph of cormorant 86's breast wound closed with sutures on 19 February 2008 (day 14 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. The wound continues to heal. The bleeding appears to have stopped, but there are some blood stains on the base of some feathers.

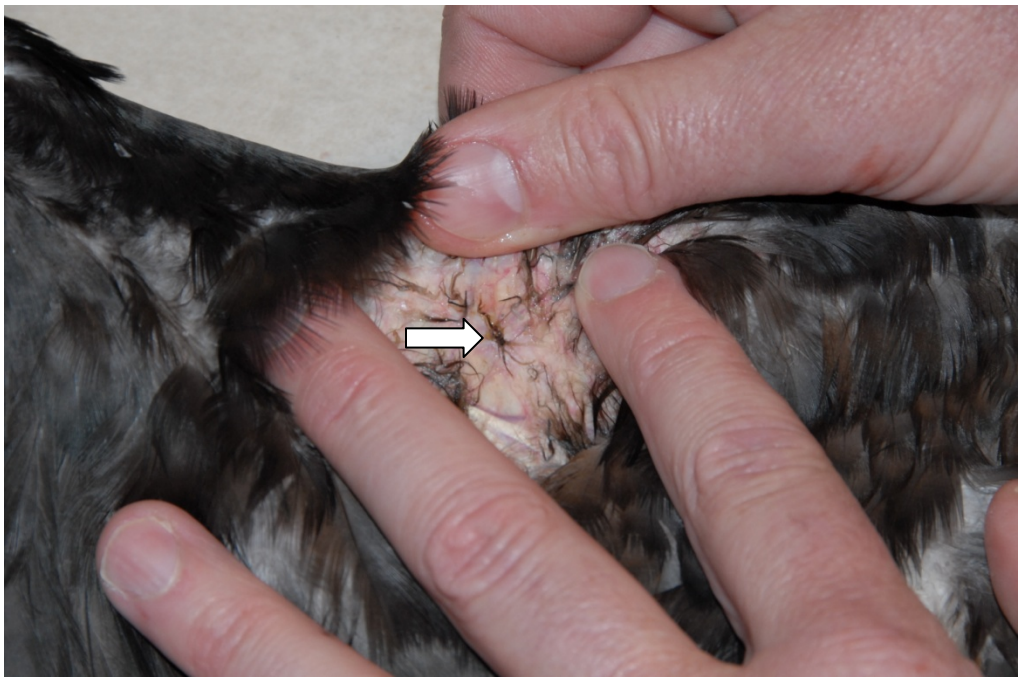

Figure 16: Photograph of cormorant 86's patagial wound closed with sutures on 19 February 2008 (day 14 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. The wound continues to heal. The feather is still caught up in the sutures.

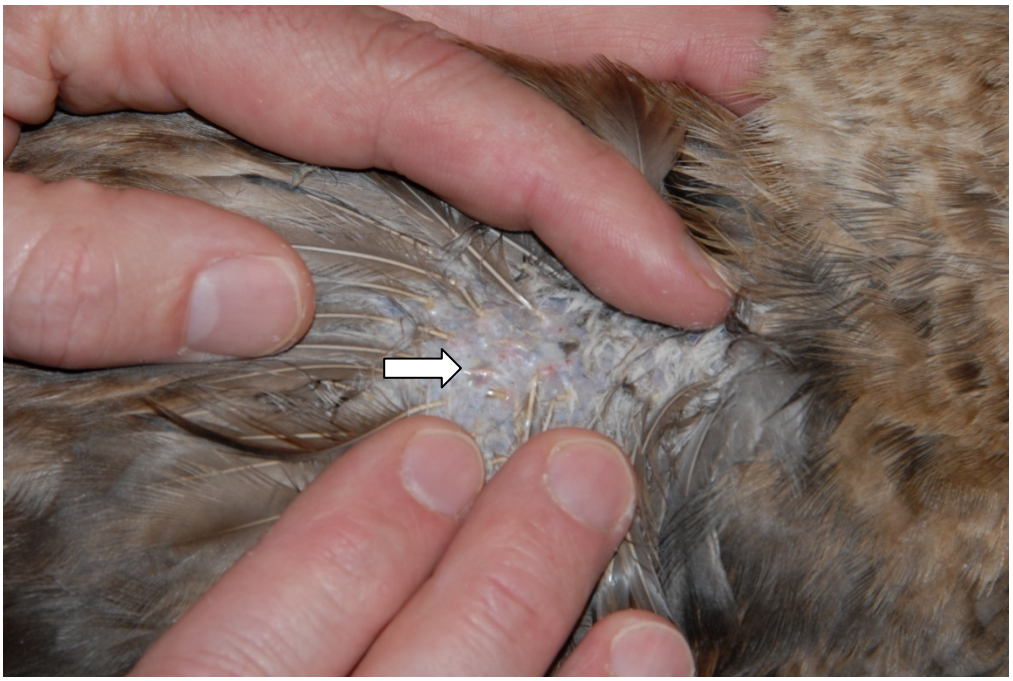

Figure 17: Photograph of cormorant 3's breast wound closed with tissue glue on 22 February 2008 (day 17 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. The skin has completely healed, and no scarring has appeared to have taken place. The remaining glue was cut away from the area to assess the wound.

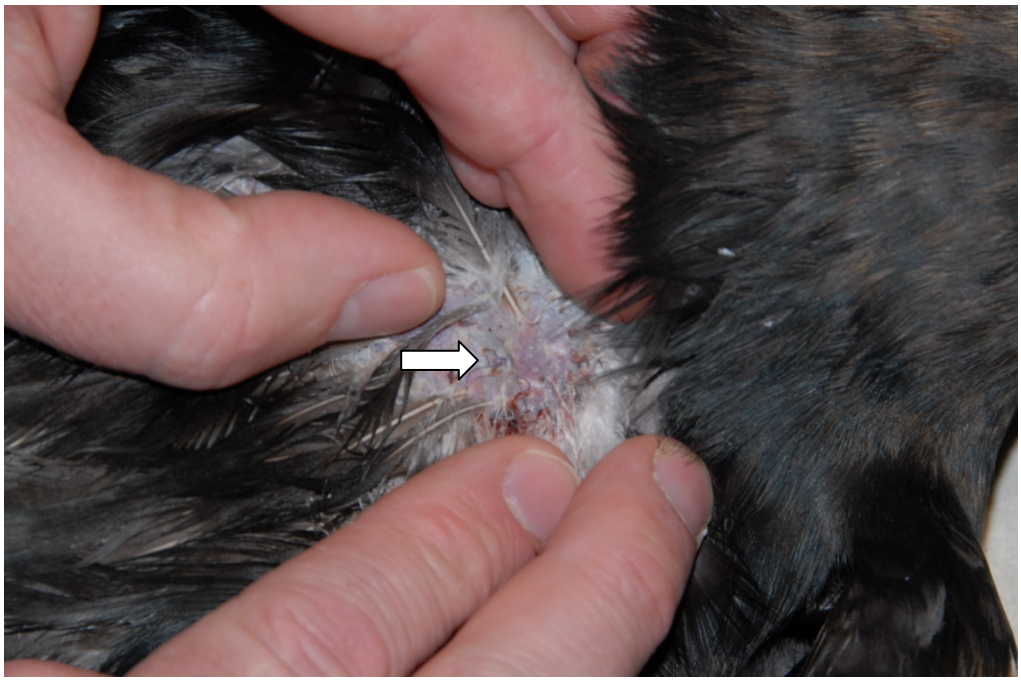

Figure 18: Photograph of cormorant 86's breast wound closed with sutures on 22 February 2008 (day 17 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. The skin has completely healed. The sutures have come loose and have nearly been pulled out (most likely from preening). There appears to be no scar where the biopsy took place. There are still some blood stains on the base of a few feathers.

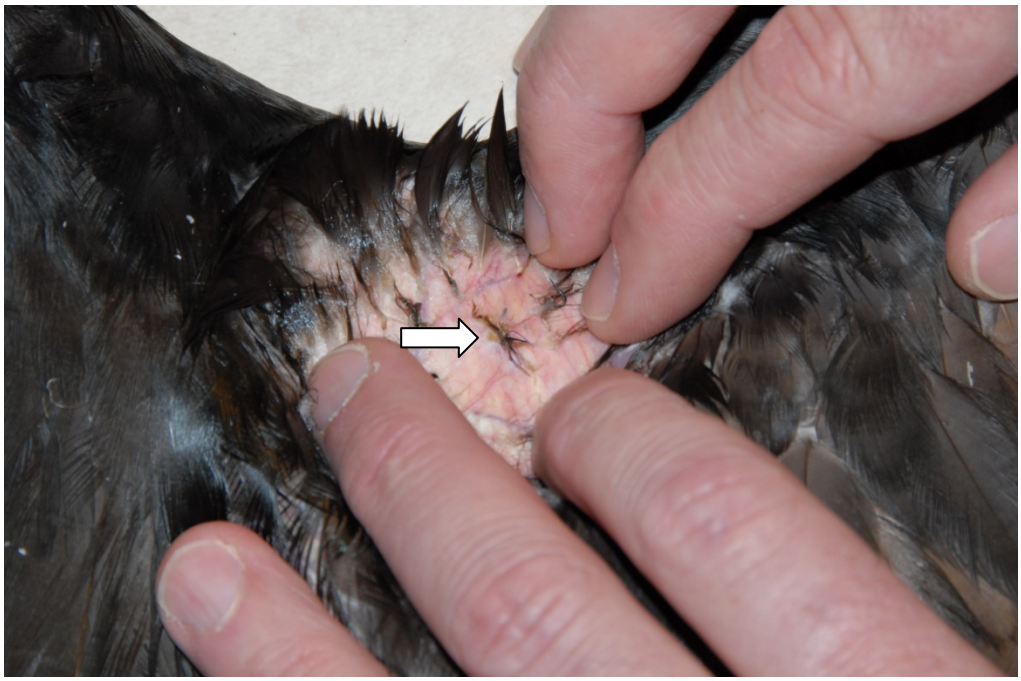

Figure 19: Photograph of cormorant 86's patagial wound closed with sutures on 22 February 2008 (day 17 post sampling) at the USDA Wildlife Services holding facility in Starkville, MS, USA. The skin has completely healed. The sutures are still in place and the feather remains caught up in the sutures.