WestVirginiaUniversity
THE RESEARCH REPOSITORY @ WVU

2004

Pentosidine as a biomarker for age in birds and museum-prepared study skins

Jesse A. Fallon
West Virginia University

Follow this and additional works at: https://researchrepository.wvu.edu/etd

Pentosidine as a Biomarker for Age in Birds and Museum Prepared Study Skins

Jesse A. Fallon

Thesis submitted to the
Davis College of Agriculture, Forestry and Consumer Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of

Master of Science
in
Animal and Veterinary Sciences

Hillar Klandorf, Chair
Kenneth P. Blemings
James T. Anderson

Department of Animal & Veterinary Sciences

Morgantown, West Virginia
2004

ABSTRACT

Pentosidine as a Biomarker for Age in Birds and Museum Prepared Study Skins

Jesse A. Fallon

The phenomenon of aging is progressive, irreversible and universal to all living things. One unavoidable outcome of aging is the modification of proteins by sugars, which, through a non-enzymatic process, can lead to the formation of crosslinked compounds termed advanced glycation endproducts. One such endproduct, pentosidine (Ps), is an indicator, or biomarker, of biological age. Because most birds have no reliable markers of age beyond adulthood, this investigation examines the accumulation of Ps as an estimator of chronological age in birds. The first study herein examines interspecies comparison of Ps accumulation in Ruffed grouse and Double-crested cormorants. The second investigates the stability of Ps in museum prepared study skins. Accurate age information can contribute to our understanding of population demographics, could aid species recovery programs, and provide insights into longevity now understood entirely from banding programs and captive birds.

Dedication

This work is dedicated to my wife, Katie Fallon. Her
unending love, inspiration, tolerance and support have made
its completion possible.

Acknowledgments

Table of Contents

Literature review

The phenomenon of aging is progressive, irreversible and universal to all living organisms. Though it is a basic, unavoidable, biological progression beginning at birth and ending only with death, its exact causes and mechanism still elude us. Despite considerable progress in the development of testable hypotheses concerning aging in an evolutionary context, a unifying theory of molecular and physiological aging has not yet been reached. As with most scientific endeavors, perhaps the mystery of aging can be unraveled piece by piece, in small steps. Like a great knot, each loop and kink must be pulled through in turn, until finally we have a cord that can be pulled straight, with a definitive beginning and end. With this approach in mind, we may begin our unraveling by examining certain cues or markers of the aging process.

On an evolutionary scale, we can use chronological age as a measure of senescence within populations. However, this may not be a reliable index of the extent of senescence in an individual because the rate of aging varies considerably among individuals within any given species (Baker and Sprott 1988). This line of reasoning has led to the concept of biological age as distinct from

chronological age, and therefore an interest in developing a means to measure biological age within an individual arose. Such a quantitative biomarker would allow not only for estimation of biological age, but, because aging increases susceptibility to disease and finally to death, also as a predictor of future occurrence of age-associated diseases, impending mortality, and maximum longevity of species (Masoro 1988).

Several potential biomarkers of age have been examined, and some of these indicators of biological age do correlate with chronological age within individuals. Among these, physical changes in long lived proteins such as collagen have been among the most promising biomarkers of aging. These physical changes in collagen occurring with increasing age are in part the result of posttranslational modifications of sugars - modifications that have been known about for quite some time (Bunn and Higgins 1981).

Maillard Reaction

In a brief 1912 paper, Louis Camille Maillard (1912) described his observation that gently heating sugars and amino acids in water resulted in a yellow-brown colored product. It seems unlikely that the scientific community of France, even in 1912, would have been surprised or

2

impressed by Maillard's results. Over the course of the

next 90 years, however, the reaction that now bears his

name proves consequential for many disciplines. Indeed,

Maillard chemistry has been intensely studied by food

scientists, physiologists, chemists, physicians and many

others.

The Maillard reaction is now understood to represent a

group of biochemical non-enzymatic browning reactions

between aldehydes, ketones, or reducing sugars and amino

acids, peptides or proteins (Monnier and Cerami 1981).

While the precise nature of Maillard chemistry has yet to

be fully defined, the immediate end-products of this

reaction are known to undergo several alterations. Early

work by Amadori (1931), Kuhn and Weygand (1937), Simon and

Kraus (1970), and Heyns et al.(1957) uncovered the initial

series of reactions in which the carbonyl moiety of the

sugar molecule reacts with a biological amine such as an

amino acid or the lysine residue of a protein forming a

Schiff base (Fayle and Gerrard 2002). This Schiff base is

unstable, and can undergo rearrangements resulting in what

is termed an Amadori product, a reasonably stable

aminoketose (Cerami et al. 1987). An example of this

portion of the Maillard reaction can be seen in Figure 1.
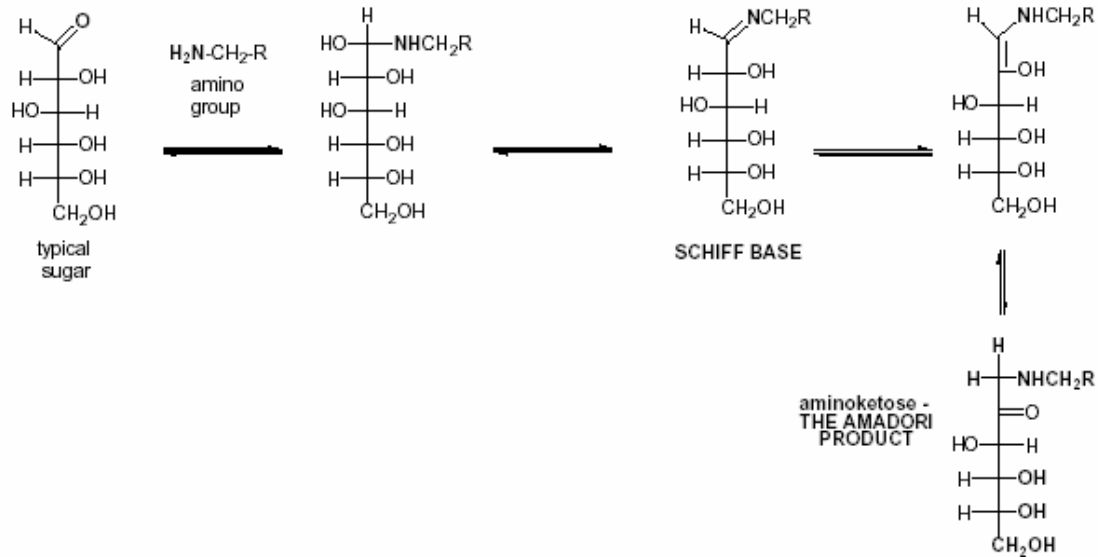
Figure 1. Maillard chemistry: sugar reacting with amino groups to form Amadori product – as presented by Fayle and Gerrard (2002).

The Amadori products can then undergo further inter- and intramolecular rearrangements. These final rearrangements produce a variety of structurally diverse compounds possessing distinctive fluorescent and crosslinking properties known as advanced Maillard products or, more commonly, advanced glycation end products (AGEs) (Monnier et al. 1984). Although Louis Camile Maillard described reactions between reducing sugars and amines more than 90 years ago, only recently did an appreciation for the formation of AGEs *in vivo* begin to fully develop.

Advanced Glycation Endproducts

Advanced glycation endproducts and AGE crosslinks are the final destinations of the Maillard reactants (Monnier et al. 1984). Found to form in all proteins, AGEs are stable, irreversible, and therefore cumulative with age *in vivo*. This accumulation is known to have adverse affects on the functionality of proteins, lipids, and DNA (Ledl and Schleicher 1990). Not surprisingly, AGEs accumulate most noticeably in long-lived tissues such as lens crystallins and collagen (Monnier and Cerami 1983; Kohn et al. 1984). In the eyes, formation of AGEs contributes to the development of cataracts, while in collagen to the yellowing and loss of elasticity, causing stiffness in joints and wrinkling of skin. Numerous studies have demonstrated the accumulation of AGEs with age in the collagen of mice, birds, and humans. Because of the cumulative nature of these compounds AGEs are now considered a biomarker of biological age (Bellmunt et al. 1995; Pokharna et al. 1995). While interest in the formation and accumulation of AGEs spans many fields, they have perhaps been most closely examined in human medicine.

Advanced Glycation Endproducts in Diabetes Research

Because of their inability to maintain stable levels of blood sugar, and the role of sugar modification in AGE formation, it was proposed that diabetics should accumulate AGEs at a greater rate than non-diabetics (Odetti et al. 1992). The physiological relevance of AGE accumulation in diabetics has been confirmed by observations revealing that antibodies prepared against AGE-proteins react with antigens in tissue proteins, that these AGE antigens increase in human lens proteins with age, and that they are present at higher age-adjusted levels in human serum (Ikeda et al. 1998; Makita et al. 1992). Additionally, diabetic rats were found to develop AGE antigens more rapidly than normal rats in aortic and renal collagen (Makita et. al. 1992). Advanced glycation endproduct accumulation therefore, may be a causative agent in some of the symptoms of late-stage diabetes (Bucala and Cerami 1992).

This line of reasoning led to examination of pathobiochemical pathways of AGE formation in hopes of uncovering interventive strategies. These investigations revealed that activated oxygen species (ROS) are involved in the formation of AGEs (Baynes 1991). The term "glycoxidation," coined by Baynes in 1991, is descriptive of the glycation and oxidation steps required for formation

of at least some AGEs. Furthermore, binding of an intracellular receptor to AGEs, termed RAGE, has been has been shown to cause depletion of cellular antioxidant-defense mechanisms as a consequence of the generation of ROS, leading to increased cellular oxidative stress (Bierhaus et al. 1997). Advanced glycation endproducts may then be biomarkers not only for age, but also for oxidative stress (Suzuki et al. 1999).

While research with human diabetics has helped to uncover the pathways to formation and structures of several of these end products, it is likely that a great many more have yet to be uncovered. The few that have been examined extensively include carboxymethl lysine (CML), pyrraline, and pentosidine (Dyer et al. 1993; Hayase et al. 1989; Sell and Monnier 1989a). Along with CML, the AGE pentosidine has been of particular interest as a biomarker for diabetic complications and aging in general (Sell et al. 1992; Sell and Monnier 1989a).

Pentosidine

Pentosidine (Ps), one of the most heavily researched AGEs, was first isolated from human dura mater in 1989, in which it was found to accumulate with age (Sell and Monnier 1989b). It is a highly fluorescent AGE crosslink

containing an arginine and lysine residue bound by a
molecule of ribose (Figure 2). While its formation can be
elicited *in vitro* from pentoses (hence its name), hexoses,
ascorbate, and a variety of Amadori products, the major
carbohydrate source *in vivo* has yet to be identified (Sell
and Monnier 1989a; Dyer et al. 1993). Again, its formation
is dependent on oxidation and is considered both an AGE and
a glycoxidation product (Baynes 1991). Although Ps
represents only a small portion total senescent
crosslinking, it is considered an excellent biomarker for
non-enzymatic modification of long-lived proteins,
providing insight into the role of the Maillard reaction in
aging and disease (Dyer et al. 1993).
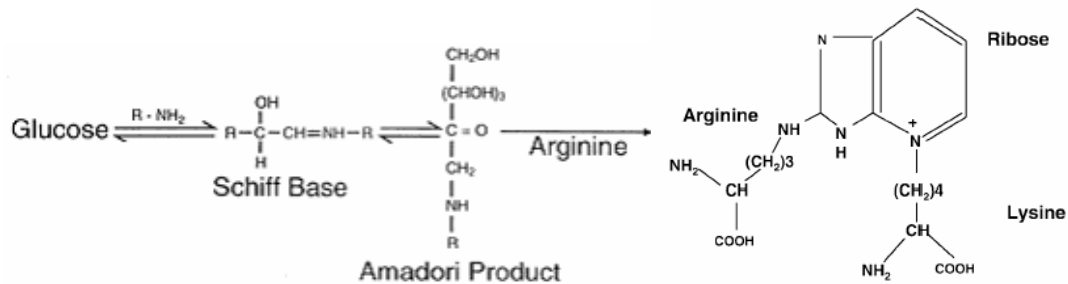
Figure 2. Proposed *in vivo* formation and structure of
pentosidine – adapted from Sell et al. 2000

Numerous studies have demonstrated the accumulation of
Ps in long-lived tissue proteins. This progressive

increase has been demonstrated in human skin, dura mater, and cartilage (Sell and Monnier 1996; Dyer et al. 1993), as well as in the skin of rodents, cows, pigs, dogs and monkeys (Sell and Monnier 1996). Furthermore, these studies suggested that Ps accumulation rates are inversely related to longevity. That is, species that tend to have shorter lifespans accumulate Ps more rapidly than those with longer maximum lifespans.

While these findings were not absolute and maximum levels of Ps between species varied greatly, they do suggest that a process that is reflected in the age-related accumulation of Ps may operate in the determination of maximum longevity. If this is the case, a strong correlation between the accumulation of AGEs like Ps and the age of individual animals should become apparent.

Indeed, the relationship between Ps accumulation and age has been shown to be strongly correlated. Sell et al. (1992) fitted the accumulation of Ps in pmol/mg collagen with age in the skin of humans with a linear regression model (R^2 = 0.73). The same laboratory has more often fitted curvilinear models to a variety of mammalian species with similar strong correlations (Sell et al. 1996). Additionally, diabetics have been shown to accumulate more Ps then non-diabetic humans, again suggesting that the lack

of metabolic control leads to increased glycation and thus an increase in AGEs (Sell et al. 1992).

It seems clear that a correlation exists between Ps accumulation in collagen and the age of individual animals, at least in mammalian species. It is difficult to imagine, however, that a similar correlation should not be present in other, non-mammalian species as well.

Pentosidine Quantification in Birds

AGEs have been quantified in a few species of birds including turkeys (Rendell et al. 1985) and hummingbirds (Beuchat and Chong 1998). Iqbal et al. (1999b) confirmed the accumulation of Ps in ad-libitum-fed broiler breeder hens. As expected, concentrations of Ps were found to increase with age. This study also suggested that birds accumulate Ps more slowly than mammals with similar metabolism, supporting the link between less rapid AGE formation and longevity. Examining a variety of avian species, Chaney et al. (2003) described a linear increase in Ps concentration (pmol/mg collagen) with age. The work also suggested that by applying this linear correlation to individual birds, the age of adult birds could be estimated.

Avian Aging

Most mammals exhibit outward morphological signs of aging which include wrinkling of exposed skin, graying of hair around the face and muzzle, tartar buildup or wear of molars, and reduction in bone density. Unlike their furry, endothermic counterparts, birds have evolved in such a way that outward signs of aging do not develop. Evolution for flight has produced skin that is covered by feathers, hollow bones, and the replacement of teeth and jawbones with a bill. While the experienced ornithologist, and in fact the novice bird watcher, can readily discern a juvenile bird from its adult counterpart through alterations in plumage color or feather wear, or by examining rectrix (tail feather) or spur length, once the threshold into adulthood has been crossed, no morphological signs of aging exist (Winker, 2000; Greenburg et al. 1972).

Current age data therefore must rely on leg or wing banding. While this is an accurate method, birds must be captured at a young, recognizable age, and then recovered at a later time - a labor intensive and rather protracted method. The technique developed by Chaney et al. (2003) using Ps accumulation as a biomarker of age, if applied to individual avian species, could prove invaluable to age estimation.

The ability to estimate age would enable researchers to make inferences concerning demographics. More than simply knowledge about average and maximum lifespans, age estimation could answer questions such as, when do different species of birds become sexually mature? How does age affect territory establishment? At what age does reproductive ability reach its peak? When do fertility rates begin to decline? Are different age groups susceptible to different diseases or pathogens? These are just a few of many lines of inquiry that could benefit from a technique for age estimation in adult birds.

Birds as Models for Aging

Beyond gaining a greater understanding of avian physiology and demographics through the application of a technique for age estimation, perhaps we can learn more about the aging process in general. All animals are born, inevitably die, and all the while they are aging. Because of the endogenous nature of aging, animal models can be used to help us better understand what factors lead to and what mechanisms cause aging.

Avian models in particular give us a unique glimpse into metabolic factors that are believed to encourage biological aging. For example, birds have overall

metabolic rates as much as 2.5 times higher than those of

mammals, with lifetime energy expenditures reaching 5 to 15

times greater (Holmes 1995, 2001). This presumably exposes

them to a higher rate of free radical production, and

should lead to accelerated tissue damage (Del Maestro

1980). Birds also maintain core body temperatures

approximately 3°C higher, as well as carrying much higher

levels of blood glucose (two- to four- fold higher) than

mammals (Holmes, 1995). These factors should, according to

current understanding, be liabilities toward biochemical

aging and lead to a more rapid accumulation of deleterious

by-products, such as AGEs, thereby rendering birds more

susceptible to age-dependant degenerative processes.

Cumulatively, these attributes should lead to a reduction

in overall life expectancy. Birds, then, should have

shorter maximum lifespans than comparatively sized mammals.

Surprisingly, the exact opposite is the case. Birds

are strikingly longer lived than mammals of comparative

size (Lindstedt and Calde 1976). Longevity records from

wild birds indicate that Mourning doves (*Zenaida macroura*)

can live to at least 31 years of age and Red-tailed hawks

(*Buteo jamaicensis*) to 29 years (Klimkiewicz and Futcher

1989). One Layson albatross (*Diomedea immutabilis*) lived

to the ripe old age of 53 (Fisher 1975). Unconfirmed

reports of Psitticines in captivity living into their 80's and 90's abound. This remarkable longevity becomes all the more fascinating when coupled with the physiology of class Aves, and in fact defies much of what we believe influences maximum lifespans.

Using birds as models for aging may help reveal biological techniques that organisms use to slow the aging process. If this is the case, perhaps these techniques could be applied to other species, humans for instance, who have had intense interest in slowing or preventing the aging process since before Ponce de Leon searched in vain for the fountain of youth. While it is unlikely that avian models can bring us to immortality, understanding how birds cope with their metabolic disabilities may contribute to the prevention of age-related diseases in humans. Moreover, because all eukaryotic organisms age, information obtained concerning aging in birds should parallel aging in other animals.

The remarkable longevity in the majority of birds does not, however, hold true for all species. Galliformes such as domestic chickens (*Gallus gallus*), Wild turkey (*Meleagris gallopavo*), and Ruffed grouse (*Bonasa umbellus*) have much shorter lifespans than those from the families Columbidae, Falconidae, Hydrobatidae, and Psitticidae.

Because data in mammals suggest that shorter-lived species accumulate AGEs more rapidly than those with greater maximum lifespans, it stands to reason that the shorter lived gallinaceous birds should show the same trait (Sell and et al. 1996). This possibility becomes exceedingly important if the biomarker Ps is to be extrapolated to an age estimate. Therefore, interspecies comparisons of Ps accumulation will be vital to the practical application of such a technique.

This accumulation of knowledge on aging, AGEs, and birds provides a firm grasp on a kink, however small, in the metaphorical knot. The following studies continue to investigate the use of Ps accumulation as a biomarker for age in adult birds. Specifically, they examine interspecies comparison of Ps concentrations, the stability of Ps in museum prepared study skins, and variations in Ps accumulation between different locations around the body.

INTERSPECIES COMPARISON OF PENTOSIDINE ACCUMULATION

AND ITS CORRELATION WITH AGE IN BIRDS

Jesse A. Fallon[1], Robert L. Cochrane[1], Brian Dorr[2], and
Hillar Klandorf[1]

[1]Division of Animal and Veterinary Sciences, West Virginia
University, Morgantown, West Virginia 26506-6108, USA
hillar.klandorf@mail.wvu.edu

[2]USDA Wildlife Services, National Wildlife Research Center,
Mississippi Field Station, P.O. Drawer 6099, Mississippi State
University, Mississippi 39762, USA

Abstract.- Recent studies suggest that a linear correlation exists between the age of individual birds and the accumulation of the advanced glycation endproduct pentosidine (Ps). This investigation compares Ps accumulation in skin tissue of Ruffed grouse (*Bonasa umbellus*) and Double-crested cormorants (*Phalacrocorax auritus*). Skin samples were taken from the breast of 52 Ruffed grouse of known ages ranging from a few days to ten years. Skin was cleaned of feathers and adipose tissue, acid hydrolyzed and measured for collagen content via hydroxyproline spectrophotometric assay. Ps concentrations were determined using reverse phase HPLC. Fluorescent detection (excitation 310nm, emission 385nm) revealed concentrations of Ps/mg collagen to increase curvilinearly with age (R^2 = .87, p<.0001). Additionally, a blind study of 19 Double-crested cormorants revealed a linear relationship between Ps concentrations and age (R^2 = .93). Comparison of these two species revealed a more rapid accumulation of Ps in Ruffed grouse. Accurate age information could aid species recovery programs and provide insights into longevity now understood entirely from banding programs and captive birds.

The phenomenon of aging is progressive, irreversible and universal to all living things. One unavoidable outcome of aging is the modification of proteins by sugars, which, through a non-enzymatic process known as the Maillard reaction, can lead to the formation of crosslinked compounds termed advanced glycation endproducts (AGEs) (Cerami et al. 1987). These protein crosslinks, like the process of aging that their formation is associated with, are irreversible. Because of this stability, AGEs are cumulative *in vivo*, most noticeably in long-lived tissues such as collagen. One such AGE, pentosidine (Ps), is a stable, fluorescent crosslink comprised of an arginine and lysine residue bound by a pentose sugar. The cumulative nature and inherent fluorescence of pentosidine make it a valuable biomarker of aging that can readily be measured in the laboratory.

Pentosidine has been found to accumulate with age in the skin collagen of a variety of mammalian species (Sell et al. 1996). Moreover, interspecies comparison of pentosidine accumulation in mammals has demonstrated that Ps accumulates at different rates in different species, and that an inverse relationship exists between maximum longevity and pentosidine (Sell et al. 1996). That is, species with shorter lifespans tend to accumulate

pentosidine more rapidly than those with greater maximum
life expectancies.

Work by Iqbal et al. (1997, 1999) confirmed the
accumulation of pentosidine with age in domestic poultry.
More recently, Chaney et al. (2003) found that pentosidine
accumulation was linearly correlated to the age of a
variety of wild bird species. These studies indicate that
the accumulation of the glycoxidation product pentosidine
is strongly correlated to the age of individual birds,
verifying it as a biomarker for the aging process (Iqbal et
al. 1999; Chaney et al. 2003). Furthermore, Chaney et al.
(2003) suggested that the linear accumulation of
pentosidine could be used as an estimate of the
chronological age of individual birds. The variability in
pentosidine concentrations associated with these data from
a variety of species, however, necessitates examination
between individual species.

Because most birds exhibit few or no external
morphological changes beyond adulthood, current information
on aging relies on banding data, a labor intensive and
protracted technique. Using pentosidine as a biomarker for
aging would provide insight into age related population
demographics such as the onset of sexual maturity, age-
specific fecundity, and the effect of age on behavior and

susceptibility to disease (Johnsen et al. 2003; Maransky and Bildstein 2002). Additionally, because many species are strong pair-bonders, species survival programs would benefit from the ability to pair non-banded birds of similar ages.

If this technique is to be used as a chronological estimate of age in birds, and, as in the mammalian models presented by Sell et al. (1996), Ps accumulation varies between individual species with different maximum lifespan potentials, an understanding of what variations exist among avian species with varying life expectancies must be attained. For this reason, we examined the potential use of pentosidine as a biomarker to estimate the chronological age in a relatively short-lived, granivorous species, the Ruffed grouse (*Bonasa umbellus*). These data are compared to a blind study which examines pentosidine accumulation in a longer-lived, piscivorous species, Double-crested cormorants (*Phalacrocorax auritus*).

MATERIALS AND METHODS

Tissue Procurement.-Grouse samples were obtained at necropsy from West Virginia University's captive colony immediately after death. Approximately 25 grams of skin was removed from the breast of 29 male and 23 female grouse

of known ages ranging from 33 days to 10 years. For up to one month prior to processing, samples were stored at -80°C.

Previously banded cormorants of known ages were collected from the wild in Mississippi and Alabama by employees of the U.S. Department of Agriculture/Wildlife Services (USDA/WS) National Wildlife Research Center, from October through April of 2002-2003 and 2003-2004. Banded cormorants also were obtained from individuals and agencies taking cormorants under the U.S. Fish and Wildlife Service unlimited depredation order. Skin samples (*n*=19) of approximately 150 grams were taken at necropsy and frozen in a sterile saline solution until assayed. Age data were not provided with the samples, which were processed immediately upon arrival.

Sample Processing.-Skin samples of approximately 2 cm^2 were prepared as described by Iqbal et al. (1997) and Chaney et al. (2003). Samples were scraped and cleaned of feather shafts, adipose tissue, and circulatory vessels. Forty mg of the remaining connective tissue was minced and then delipidated in a 2:1 chloroform/methanol solution for 18 hours. Following delipidation, the residue was recovered by centrifugation and placed in 4 ml of 1:1

water/methanol solution for 2 hours. Again the residue was
recovered by centrifugation, and then acid hydrolyzed in
4ml of dearated 6 N HCL for 18 hours. The acid was
evaporated by a Speed-Vac (Savant Instruments, Farmingdale,
New York), before reconstituting the residue with 500 µl of
water. Each sample was then filtered through a Costar
Spin-X centrifuge tube filter.

Collagen and Pentosidine Measurement.-Collagen content
was determined via spectrophotometric hydroxyproline assay,
assuming 14% of collagen by weight to be hydroxyproline
(Maekawa et al. 1970).

Measurement of Ps concentration was accomplished via
slight modification of techniques used by Iqbal et al.
(1999) and Chaney et al. (2003). Pentosidine
concentrations were estimated using a Waters 2690 HPLC work
station with a Waters 494 in-line fluorescence detector
(excitation 310nm, emission 385nm). Elutions off the C-18
column (YMC OCS-AQ 4.6 x 250 mm) were obtained using a
linear gradient of 5-28% acetonitrile in water with .01 M
heptafluorobutyric acid from 0 to 35 minutes. The column
was cleaned and equilibrated for 18 and 12 minutes
respectively. Pentosidine concentrations in duplicate
samples spiked with known amounts of Ps also were measured

to ensure accurate elution time. Quantification of Ps concentration was accomplished using a Ps standard. Integration of peaks was accomplished with Millennium 32 version 3.05.01 software package.

Statistical regression analysis and comparison was completed using SAS software (SAS Institute, Cary, North Carolina). Methods of analysis included Proc GLM, Proc REG, and SAS Proc NLIN.

<div align="center">RESULTS</div>

The concentration of Ps was found to increase with age in both species of birds.

Ruffed grouse.-Regression analysis of 52 grouse skin samples revealed a curvilinear increase of Ps in ruffed grouse with $y = 0.0059x^2 + 0.1551x + 11.636$, $R^2 = 0.87$ (p<0.0001) (Fig. 1). Grouse data were also fitted with the linear regression line $y = 0.8063x + 2.1542$, $R^2 = 0.80$ (t = 15.20, p<0.0001). For further analytical comparisons, this relationship, having only a modest effect on the coefficient, will be considered for ease of interspecies comparison. No significant sex differences were detected using either analysis.
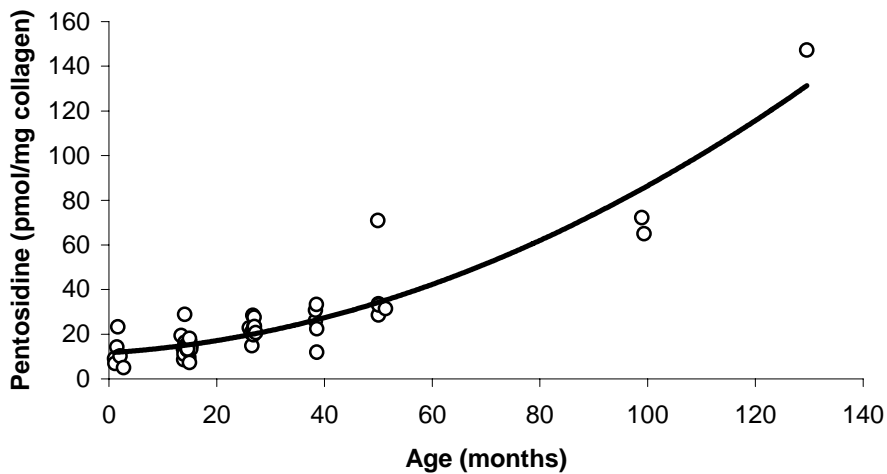
Figure 1. Pentosidine concentration as a function of age in Ruffed grouse.

Double-crested Cormorants.-Pentosidine data from the blind study of 19 cormorants revealed two distinct age groups. This prediction, along with the identification of what appeared to be the 4 oldest and 7 youngest birds, was verified *a-posteriori* from banding data acquired from the U.S. Geological Survey Bird Banding laboratory. Each of these predictions was independently found to be accurate, at which point age data were provided to our laboratory.

Pentosidine concentrations were found to increase linearly with age in cormorants, with $y = 0.1914x + 6.6701$, $R^2 = 0.93$ ($t = 15.40$, $P < 0.0001$) as seen in Figure 2.
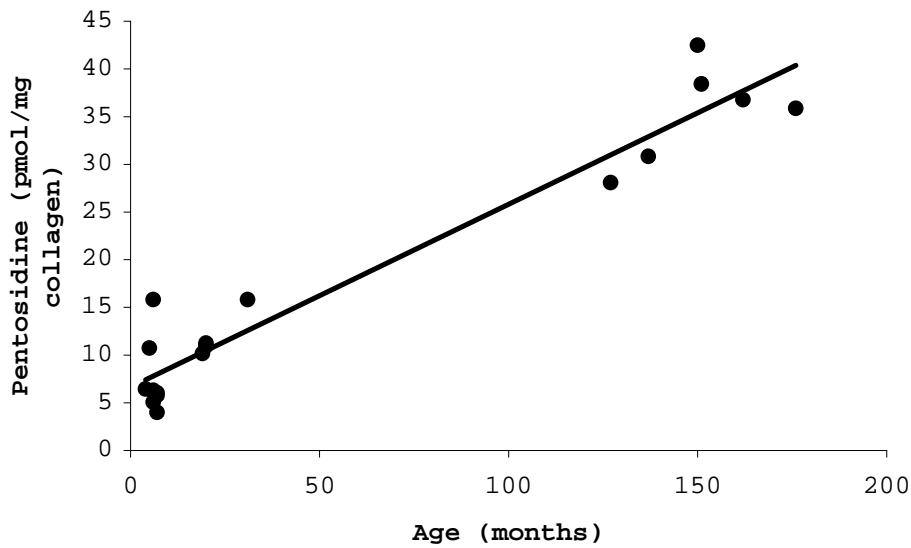
Figure 2. Pentosidine concentration as a function of age
in Double-crested cormorants.

Interspecies comparison.-Comparison between species

revealed a more rapid increase in Ps concentrations in

grouse than in the longer-lived cormorants (Fig. 3). Slope

analysis of the regressions confirmed a significant

difference in Ps concentrations (t=11.03, P<0.0001).

Additionally, comparison of the regression line from Chaney

et al. (2003) revealed a slope very similar to the

cormorant data, and different from the grouse data (Fig.

3).
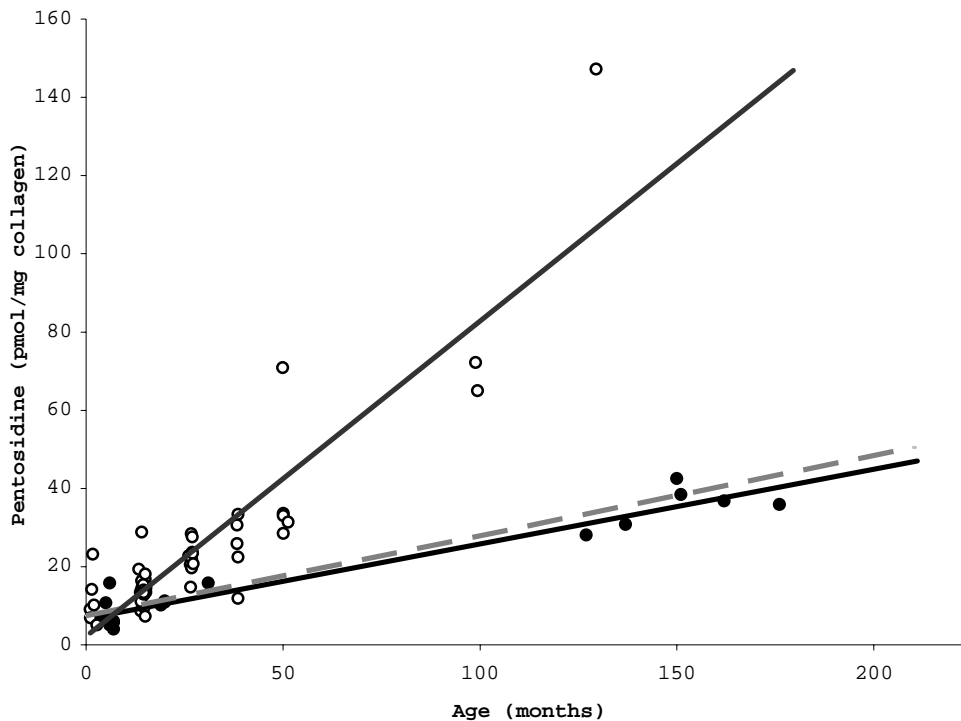
Figure 3. Pentosidine concentration as a function of age
in Ruffed grouse (open circles) and Double-crested
cormorants (closed circles). Dotted line represents
results from Cheney et al. (2003).

DISCUSSION

In agreement with previous research, Ps accumulation
was found to increase with age in both Ruffed grouse and
Double-crested cormorants. Ultimately, the goal of this
work is to measure Ps, and, using these established
relationships, infer the unknown ages of individual birds.

Data from the blind study of cormorants, which led to an accurate prediction of the oldest, youngest, and similarly aged birds, demonstrates the power of this technique. The ability to categorize birds into age groups alone, even without an established relationship between Ps accumulation and age in an individual species, can contribute enormously to information concerning age related affects on fertility, behavior, and susceptibility to disease.

Although a curvilinear relationship provided the best fit for the grouse data, this relationship depended on Ps concentration in a particularly aged, captive reared grouse which was likely approaching the maximum theoretical lifespan of this species at 10 years of age. More data from grouse of similar age may help confirm this curvilinear relationship. It is possible that as individuals reach extreme geriatric age, the formation of AGEs like Ps accelerate dramatically due to impaired collagen turnover or an increase in oxidative damage leading to age-related physiological malfunctions such as end stage renal disease, as has been suggested in humans (Sell and Monnier 1990).

While a curvilinear relationship between age and Ps concentration was not supported by the cormorant data (fig.

2), the lack of individuals of intermediate ages may have affected slope estimates for this species. Additional data from "middle-aged" cormorants may indicate a curvilinear relationship more similar to that of the grouse, although the less rapid accumulation of Ps in this longer-lived species would remain.

The distinct difference between Ps accumulation in grouse and cormorants, as illustrated in fig. 3, could be attributed to several factors. First, even in the wild, these two species have vastly different diets and thrive in entirely different habitats. Second, while these Ruffed grouse were raised in captivity and fed *ad-libitum*, the cormorants had to rely on their foraging abilities in the wild. In mice, dietary restriction has been demonstrated to lead to a decrease in the accumulation of Ps, as well as an increase in longevity (Sell et al. 2000). The difficulty in finding food in the wild when compared to the unlimited access in a captive setting may represent a situation of comparative dietary restriction.

It seems more likely however, that these differences are due to overall longevity of the individual species as suggested in mammals (Sell et al. 1996). *Galliformes* are a relatively short-lived family of birds, with the oldest wild Ruffed grouse on record being 8½ years of age

(Klimkiewicz and Futcher 1989). *Pelecaniformes* tend to

live much longer, with the oldest wild Double-crested

cormorant in North America reaching nearly 18 years

(Klimkiewicz and Futcher 1989). Although the underlying

mechanisms of the association between Ps accumulation and

longevity remain to be established, an inverse relationship

between the two has been demonstrated in a variety of

mammalian species (Sell et al. 1996).

Whether diet, habitat, longevity, or a combination of

the three contribute to these differences in Ps

accumulation, it seems unlikely that a generic relationship

between the age of individual birds and Ps accumulation

will be applicable to all species. However, the striking

similarity between the relationship found for the variety

of species by Chaney et al. (2003) and the cormorants in

this study suggests that some overlap may exist (Fig. 3).

Perhaps Ps accumulation between closely related species or

species with similar lifespans will coincide enough to make

some generalizations. Further investigation into species,

family, and genera specific models are needed to confirm

this.

Although these data present some variation in age

estimates, it seems likely that, using Ps accumulation as a

guide, we can make accurate age estimates of individual

adult Ruffed grouse and Double-crested cormorants. As we
continue to refine the technique, examine the differences
between individual species, habitat, and diet, and reduce
the size of skin samples necessary for analysis, we will be
able to make more precise estimates of the ages of birds.

LITERATURE CITED

Cerami, A., Vlassara, J., and M. Brownlee. 1987. Glucose
    and aging. Science 256:90-96.

Chaney, R. C., Blemings, K. P., Bonner, J., and H.
    Klandorf. 2003. Pentosidine as a measure of
    chronological age in wild birds. Auk:394-399.

Iqbal, M., Probert, L.L., and H. Klandorf. 1997. Effect
    of dietary aminoguanidine on tissue pentosidine and
    reproductive performance in broiler breeder hens.
    Poultry Science. 76:1574-1579.

Iqbal, M., Probert, L.L., Al-humadi, N.H., and H. Klandorf.
    1999. Protein glycosylation and advanced glycosylation
    endproducts (AGEs): An avian solution. Journal of
    Gerontology: Biological Science 54:B1-B6.

Klimkiewicz, M. K. and A. G. Futcher. 1989. Longevity
    records of North American Birds: Supplement I. Journal
    of Field Ornithology 60(4):469-494.

Maekawa T., Rathinasamy T. K., Altman K. I., and W. F.
    Forbes. 1970. Changes in collagen with age. The
    extraction of acid soluble collagens from the skin of
    mice. Experimental Gerontology 5:177-186.

Sell, D. R. and V. M. Monnier. 1990. End-stage renal
    disease and diabetes catalyze the formation of a
    pentose-derived crosslink from aging human collagen.
    Journal of Clinical Investigation 85:380-384

Sell, D. R., Lane, M., Johnson, W. A, Masoro, E. J., Mock, O. B., Reiser, K. M., Fogarty, J. F., Cutler, R. G., Ingram, D.K., Roth, G. S., and V. M. Monnier. 1996. Longevity and the genetic determination of collagen glycoxidation kinetics in mammalian senescence. Proceedings of the National Academy of Science, USA 93:485-490.

Sell, D. R., N. R. Kleinman and V. M. Monnier. 2000. Longitudinal determination of skin collagen glycation and glycoxidation rates predicts early death in C57BL/6NNIA mice. The FASEB Journal 14:145-156.

Terres, J. K. 1980. The Audubon Society Encyclopedia of North American Birds. Alfred A. Knopf, New York, NY.

STABILITY OF PENTOSIDINE CONCENTRATIONS IN MUSEUM

PREPARED STUDY SKINS

Jesse A. Fallon[1], William J. Radke[2], and Hillar Klandorf[1]

[1]Division of Animal and Veterinary Sciences, West Virginia
University, Morgantown, West Virginia 26506-6108, USA
hillar.klandorf@mail.wvu.edu

[2]University of Central Oklahoma, Edmond, OK, 73034, USA

Abstract.— Accumulation of the advanced glycation endproduct pentosidine (Ps) in skin collagen is strongly correlated with the age of adult birds, allowing age estimates to be made without the use of banding data. The current investigation advances the use of this technique to determine the stability of Ps in museum prepared study skins. Skin samples from six Ruffed grouse prepared as study skins were taken at three month intervals over the course of one year. The data revealed no significant variation in Ps concentrations within individual birds, indicating that Ps is stable in study skins for at least twelve months. These data suggest that inferences concerning age may be made on similarly prepared specimens from collections around the world. Additionally, significant variations in Ps concentrations with respect to 6 different body locations in skin collagen of six Ruffed grouse were uncovered. This suggests that care and consistency should be employed when excising skin for Ps measurement and age estimation in birds.

Most species of birds exhibit no external morphological indicators of age once the threshold into adulthood has been crossed. The inherent value of age information and the limitations of banding data have led to

the development of a new technique for estimating the age of adult birds. It uses the accumulation of the protein crosslink pentosidine (Ps) as an indicator, or biomarker, for biological age (Sell et al. 1996; Iqbal et al. 1999; Chaney et al. 2003).

Pentosidine results from the modification of proteins by reducing sugars in a processes termed glycoxidation (Baynes 1991). An unavoidable outcome of the aging process, Ps is a fluorescent, irreversible compound that is cumulative *in vivo*. These facts make it a reliable, readily measured biomarker of aging. Accumulation of Ps has been significantly correlated to chronological age in mammals, and more recently birds (Sell et al. 1996; Iqbal et al. 1999). Chaney et al. (2003) described a linear correlation between Ps accumulation in skin collagen and chronological age in adult birds; i.e., Ps accumulates in a predictable manner. This work suggests that by measuring the concentration of Ps and using this correlation, an accurate estimate of chronological age of non-banded birds can be made (Chaney et al. 2003).

Currently, age estimation in birds using this technique has been gathered only from recently deceased specimens. It may be possible, however, to retrieve age information from another source. Ornithologists have long

been collecting birds and preparing them as museum specimens by removing and drying the skin (Winker 2000). These skins provide valuable information about morphological measurements and identification techniques for individual species (Baldwin et al. 1931). This investigation advances the potential of Ps as an estimator for age in museum prepared study skins. It examines the effect of time on the stability of Ps concentrations in Ruffed grouse (*Bonasa umbellus*) prepared as study skins, and takes multiple samples from individual birds. Stability of Ps would allow for age estimation at the time of death of specimens similarly prepared.

Consistently different levels of Ps concentration between fresh samples taken from foot webbing and breast skin have previously been noted in California gulls (*Larus californicus*) (Chaney et al. 2003). Because of these results, and because in this study multiple samples have been taken from individual study skins, we have examined the variation of Ps concentration between different body locations of individual grouse.

MATERIALS AND METHODS

Tissue Procurement and Study Skin Preparation.-Initial Ruffed grouse skin samples were obtained at necropsy from

West Virginia University's captive colony immediately after death. Approximately 25g of skin was removed from the breast of six male Ruffed grouse of known ages, two from each of three different age groups; 2, 4 and 8 years old. For the duration of the study (up to 12 months depending on what month the sample was taken) and prior to processing, samples were stored at -80°C.

After excising initial skin samples and making appropriate individual measurements (Baldwin et al. 1931), each bird was prepared as described by Winker (2000) with a few modifications. Skin was carefully removed from the six birds, cleaned of adipose and muscle tissue, and treated with hydrous sodium borate (Borax). The skins were then sewed onto cotton "bodies" attached to 3cm dowel rods for stability. These skins were allowed to air dry in a well ventilated location, and were rotated every third day to encourage even drying.

Two cm^2 samples were removed from skin around the breast of the specimens every three months over the course of one year, for a total of five chronological samples from each bird. After removal, samples were scraped and cleaned of feather shafts, adipose tissue and excess Borax. Individual skin samples were stored at -80°C for the duration of the study. Sample processing and Ps

measurements as described below began only after all samples were collected.

In a separate investigation, skin samples were taken from 6 different body locations of 6 male Ruffed grouse of known ages. These locations included the breast, abdomen, lower back, upper back, right wing, and right thigh. Samples were taken at necropsy immediately after death, cleaned of feather shafts, adipose tissue, and circulatory vessels and processed and assayed together as described below.

Sample Processing.-Skin samples were further prepared as described by Iqbal et al. (1997) and Chaney et al. (2003). Forty mg of the remaining connective tissues were minced and then delipidated in a 2:1 chloroform/methanol solution for 18 hours. Following delipidation, the residue was recovered by centrifugation and placed in 4 ml of 1:1 water/methanol solution for 2 hours. The collagen residue was again recovered by centrifugation, and then acid hydrolyzed in 4ml of dearated 6 N HCL for 18 hours. The acid was evaporated by a Speed-Vac (Savant Instruments, Farmingdale, New York), and each sample was reconstituted with 500 µl of water. Each sample was then filtered through a Costar Spin-X centrifuge tube filter.

Collagen and Pentosidine Measurement.-Collagen content of

the remaining solutions was determined via

spectrophotometric hydroxyproline assay in duplicates,

assuming 14% of collagen by weight to be hydroxyproline

(Maekawa et al., 1970).

Measurement of Ps concentration was accomplished via

slight modification of techniques used by Iqbal et al.

(1999) and Chaney et al. (2003). Pentosidine

concentrations were estimated using a Waters 2690 HPLC work

station with a Waters 494 in-line fluorescence detector

(excitation 310nm, emission 385nm). Elutions off the C-18

column (YMC OCS-AQ 4.6 x 250 mm) were obtained using a

linear gradient of 5-28% acetonitrile in water with .01 M

heptafluorobutyric acid from 0 to 35 minutes. The column

was cleaned and equilibrated for 18 and 12 minutes

respectively. Pentosidine concentrations in duplicate

samples spiked with known amounts of Ps also were measured

to ensure accurate elution time. Quantification of Ps

concentration was accomplished using a Ps standard.

Integration of peaks was accomplished with Millennium 32

version 3.05.01 software package.

Statistical Analysis.-Study skin data were analyzed using a random complete block design with months after study skin preparation serving as treatments within individual birds. Fisher's LSD test also was used to examine differences between individual birds and age groups.

Random complete block design and Fisher's LSD analysis were implemented to assess differences in Ps concentrations between 6 different body locations (treatments) of fresh samples from 6 Ruffed grouse of known ages (blocks). All data were analyzed using SAS statistical software (SAS Institute, Cary, North Carolina).

<center>RESULTS</center>

Study Skins.-Pentosidine concentrations measured in pmol/mg collagen remained stable over time in the six Ruffed grouse study skins. Randomized complete block analysis revealed no significant difference (F=1.49, P>0.24) in Ps concentrations between samples taken at three month intervals, over the course of one year, within individual birds and individual age groups. Fisher's LSD (t=2.09) uncovered no significant difference within individual age groups. The eight year old age group had significantly higher Ps concentrations than the four and two year old groups. Similarly the four year old group had

significantly higher Ps concentrations than the two year
old group.

Mean and standard deviations for each bird are listed
in Table I. Data points, mean [Ps]/mg collagen, and 95%
confidence intervals for each age group are shown in Figure
1. No trend towards a decline or increase in Ps with time
after study skin preparation was noted.

Table I.
Pentosidine stability in study skins

Means and standard deviations of pentosidine
concentrations in 2, 4, and 8 year old Ruffed grouse
different letters indicate significant difference

Age (months)	Mean Ps concentration (pmol/mg collagen)	Standard Deviation
24	22.5 [A]	2.0
24	26.7 [A]	1.5
48	34.8 [B]	2.0
48	36.1 [B]	3.8
96	70.9 [C]	5.9
96	66.3 [C]	5.0
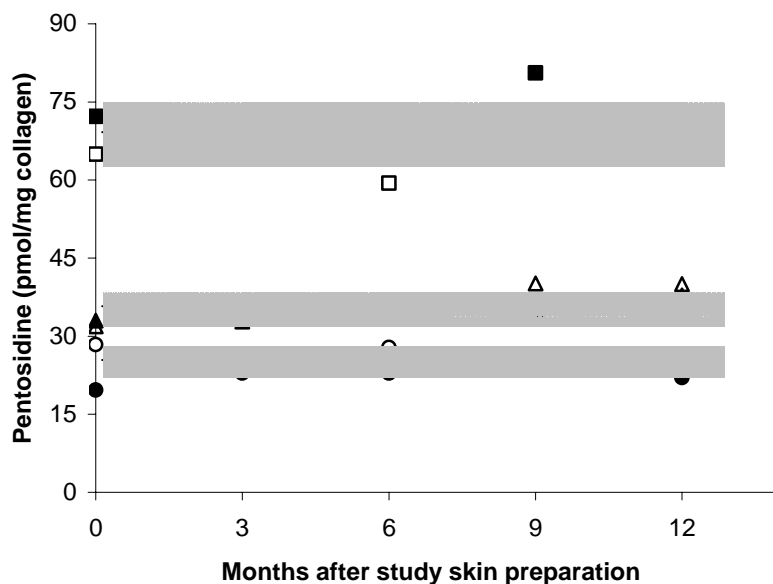
Figure 1. Mean and 95% confidence intervals of pentosidine concentration as a function of time after study skin preparation in 2 year old (circles), 4 year old (triangles), and 8 year old (squares) Ruffed grouse.

Variability Between Body Locations.-Random complete block analysis revealed significant differences between skin taken from different body locations (F=4.94, P<.01) of six grouse. Fisher's LSD test (t=2.06) confirmed skin taken from the thigh and wing area to have significantly higher concentrations of Ps then skin taken from the breast. Tissue taken from the abdomen, and the upper and lower back did not have significantly different Ps concentrations than tissue taken from the breast (table II).

Table II.
Pentosidine variation between body locations
Mean pentosidine concentrations of skin samples taken
from different body locations of Ruffed grouse
*indicates significant difference from skin taken from the breast

Body Location	Mean pentosidine concentration (pmol/mg collagen)
Breast	29.178
Abdomen	27.543
Lower Back	32.564
Upper Back	32.017
Thigh	34.795*
Wing	38.821*

DISCUSSION

In agreement with previous studies, statistical

analysis revealed Ps concentrations to be proportionately

higher in 8 year old birds than in 4 year old birds, and

similarly in 4 year olds than in 2 year old birds Table I).

Moreover, these data suggest that Ps remains stable over

time in museum prepared study skins, at least for the

duration of the sampling period of one year. Most

importantly, there was no consistent trend of Ps over time

in any of the samples (fig. 1).

Advanced glycation endproducts have been demonstrated

to have half-lives of 117 years in cartilage collagen and

15 years in skin collagen of humans (Verzijl et al., 2000).

These half-lives were determined from collagen turnover *in

vivo*. Providing proper preservation and storage, and

barring collagen degradation, Ps is likely to survive much longer in study skins. If Ps continues to maintain its stability for extended periods of time, then the prospect of obtaining samples from an enormous number of museum specimens becomes available. Indeed, by taking only a small skin sample, the ability to retrieve age information from species that have undergone extinction becomes conceivable. Specimens of Ivory-billed woodpeckers (*Campephilus principalis*), Carolina parakeets(*Conuropsis carolinensis*), and Passenger pigeons (*Ectopistes migratorius*) could provide data on the age of the animal at the time of collection.

Variations, demonstrated by standard deviations (Table I), in Ps concentrations within individual birds may possibly be due to imperfections in the technique. It seems more likely, however, that Ps concentrations in skin collagen differ between certain locations of body due to varying vascularlization, relative body temperature, rates of collagen turnover, or concentrations of tissue antioxidants. While every effort was made to obtain samples from the same general area around the breast, initial preservation and previous sample removals made this a challenging undertaking, and some samples included tissue from the abdominal region.

The variation in Ps concentrations found between different body locations in fresh samples supports this. Fortunately, the consistent differences shown here indicate that, with enough samples, perhaps compensations for different body locations may be made. In the meantime, this preliminary research implies that great care must be taken when excising skin for age estimation in both recently deceased birds and study skins.

While it is undetermined whether Ps concentrations will remain stable for more extended periods of time in museum prepared study skins, this investigation suggests relative stability for the duration of one year (Fig. 1). These data provide experimental evidence that Ps accumulation cannot only suggest age information from live or recently deceased birds, but, providing appropriate preservation, also from individuals that have expired and been prepared as study skins.

Museum specimens are admired by visitors the world over, and study skins serve as a valuable tool for ornithologists to learn identification diagnostic techniques, gather useful morphology measurements, and even extract genetic information (Winker 2000; Baldwin et al. 1931; Payne 2003). The stability of the biomarker Ps demonstrated here is unique, and these data suggest that

avian study skins can now be evaluated for age estimates as well, at least for the duration of one year. Further studies are needed to determine the long-term stability of Ps in museum specimens.

Literature Cited

Baldwin, S.P., Oberholser, H.C., and L. G. Worley. 1931.
Measurements of birds. Scientific Publication
Cleveland Museum of Natural History II.

Baynes, J. W. 1991. Role of oxidative stress in development
of complications in diabetes. Diabetes 40:405-412.

Cerami, A., Vlassara, J., and M. Brownlee. 1987. Glucose
and aging. Science 256:90-96.

Chaney, R. C., Blemings, K. P., Bonner, J., and H.
Klandorf. 2003. Pentosidine as a measure of
chronological age in wild birds. Auk:394-399.

Fayle, S. E. and J.A. Gerrard. 2002. Pages 1-8 in The
Maillard Reaction (P. S. Beldon, Ed.) Royal Society of
Chemistry, Great Britain.

Greenberg, R. E., S. L. Etter, and W. L. Anderson. 1972.
Evaluation of proximal primary feather criteria for
aging wild pheasants. Journal Wildlife Management
36:700-705.

Iqbal, M., Probert, L.L., Al-humadi, N.H., and H. Klandorf.
    1999. Protein glycosylation and advanced glycosylation
    endproducts (AGEs): An avian solution. Journal of
    Gerontology: Biological Sciences 54:B1-B6.

Iqbal, M., Probert, L.L., and H. Klandorf. 1997. Effect
    of dietary aminoguanidine on tissue pentosidine and
    reproductive performance in broiler breeder hens.
    Poultry Science 76:1574-1579.

Johnsen, A., Lifjeld, J. T., and C. Krokene. 2003. Age-
    Related Variation in Mate-Guarding Intensity in the
    Bluethroat (*Luscinia svecica*). Ethology 109:147— 158.

Kohn, R. R., Cerami, A., and V. M. Monnier V.M. 1984.
    Collagen aging in vitro by non-enzymatic glycosylation
    and browning. Diabetes. 33:57-59.

Maekawa T., Rathinasamy T. K., Altman K. I., and W. F.
    Forbes. 1970. Changes in collagen with age. The
    extraction of acid soluble collagens from the skin of
    mice. Experimental Gerontology 5:177-186.

Maransky, B. P. and K. L. Bildstein. 2002. Follow Your
    Elders: Age-related Differences in the Migration
    Behavior of Broad-winged Hawks at Hawk Mountain
    Sanctuary, Pennsylvania. The Wilson Bulletin 113:350–
    353.

Monnier V.M., and Cerami A. 1983. Detection of nonenzymatic
    browning products in the human lens. Biochemica
    Biophysical Acta. 760:97-103.

Monnier V. M., Kohn R. R., and A. Cerami A. 1984.
    Accelerated age-related browning of human collagen in
    diabetes mellitus. Proceedings of the National
    Academy of Sciences of the United States of America
    81:583-587.

Payne, R.B. and M.D. Sorenson. 2003. Museum collections as
    sources of genetic data. Bird Collections in Europe:
    the Challenge of Mutual Cooperation. Bonner
    Zoologische Beiträge 51:97-104.

Pickering, S.P.C. 1988. Attendance patterns and behaviour
    in relation to experience and pair-bond formation in

the Wandering Albatross *Diomedea exulans* at South
Georgia. *Ibis* 131: 183-195.

Sell, D.R., and Monnier, V.M. 1989. Structure elucidation
of a senescence cross-link from human extracellular
matrix. Implication of pentoses in the aging process.
Journal of Biological Chemistry 264:21597-21602.

Sell, D. R., Lane, M., Johnson, W. A, Masoro, E. J., Mock,
O. B., Reiser, K. M., Fogarty, J. F., Cutler, R. G.,
Ingram, D.K., Roth, G. S., and V. M. Monnier. 1996.
Longevity and the genetic determination of collagen
glycoxidation kinetics in mammalian senescence.
Proceedings of the National Academy of Sciences, USA
93:485-490.

Smith, C. M., Cooke, F., Robertson,G. J., Goudie, R. I.,
and W. S. Boyd. 2000. Long-term pair bonds in
Harlequin ducks. The Condor 102:201-205.

Verzijl., N., Degroot, J., Thorpe, S.R., Bank, R.A., Shaw J.N., Lyons, T.J., Bijlsma, J.W.J., Lafeber, F.P.J., Baynes, J.W., and J. M. TeKoppele. 2000. Effect of collagen turnover on the accumulation of advanced glycation end products. Journal of Biological Chemistry 275:39027-39031.

Winker, K. 2000. Obtaining, preserving, and preparing bird specimens. Journal of Field Ornithology 71:250-297.

.
Conclusions

This research confirms the use of a powerful new tool for estimating the age of adult birds. It demonstrates that the advanced glycation endproduct pentosidine is an accurate biomarker of chronological age in birds. Interspecies examination uncovered a more rapid accumulation of pentosidine in the granivorous, short lived species, Ruffed grouse than the piscivorous, long-lived species, Double-crested cormorants, supporting previous evidence of an inverse relationship between glycoxidation rate and maximum life expectancy. Additionally, the blind study of Double-crested cormorants verifies, even without previous pentosidine data, that this technique can be used to gather useful age information.

The data herein also suggest that age information can be collected from museum prepared study skins, as pentosidine was shown to remain stable in study skins for at least the duration of one year. If the crosslink continues to remain stable, this opens the door to an enormous amount of skins, potentially from birds that have gone extinct, that could be used to gather age information.

As with most scientific research, this work brings about as many questions as it does answers. Will diet and

habitat be found to impact Ps accumulation? Will maximum

lifespan be the determining factor? Can this technique be

performed on live birds in the wild without endangering

them? Will pentosidine continue to remain stable over

extended periods of time in study skins? As this project

continues, these questions are just a few of the possible

lines of inquiry. Hopefully, this current work will aid in

our understanding of the population demographics of birds,

as well as increase our awareness of birds as animal models

for aging, thereby contributing to our understanding of the

aging process in general.

It seems remarkable that an innate biological process

so entwined in the life of all multicellular organisms can

continue to confound our most diligent efforts. However,

as determined scientists, we will continue to plod along,

working at the complex knot that is the mystery of aging.

Perhaps the resilience of the problem, along with its

profound implication on the lives of us all, is what draws

us out, beckons us to pull this loop, to loosen that kink.

While the ultimate impact of this development in age

estimation of birds may be small, it is not insignificant,

and hopefully, is a step in the right direction.

Appendix A: Ps concentrations in Ruffed grouse

Bird #	Age (days)	Sex	Ps (pmol/mg collagen)
99-2	51	f	23.21240652
01-6 Blue	63	f	10.1820174
91-1	3943	m	147.2210874
01-46 green	45	f	14.16094516
99-29	409	f	19.31780863
01-13 yellow	84	f	5.069414607
01-43 yellow	35	f	6.858875355
99-13	33	f	9.094951039
98-33	1524	m	28.52789965
00-43	808	f	14.75203462
98-35	1524	m	33.60901179
94-53	3011	m	72.20836828
00-31	815	m	22.07057855
00-80	805	f	20.55311792
00-06	829	m	20.71988628
00-74	794	f	22.77633673
00-14	823	m	23.40093885
99-26	1167	m	30.65953818
01-06 yellow	437	f	13.00828168
99-12	1174	f	22.4425849
94-93	3024	m	64.94534419
01-15 green	451	f	9.554317173
01-13 blue	424	m	11.02662448
99-27	1167	f	25.92517856
98-01	1563	m	31.36841482
98-30	1525	m	33.02055565
01-15 blue	423	f	8.658886279
01-12 blue	429	m	28.80639994
01-36 green	444	m	14.04217897
00-21	816	m	19.64351154
99-18	1174	m	11.91743848
01-24 orange	450	?	13.43833861
01-28 green	456	m	18.12433242
00-28	821	m	23.64659186
01-41 green	448	f	12.78061835
01-10 green	456	m	7.306903243
01-1 yellow	443	m	15.41568936
01-74 yellow	463	f	14.96940912
01-80 yellow	463	f	14.45168527
01-33 yellow	436	m	14.90692115
01-34 blue	445	m	12.90489785
01-73 yellow	463	f	13.3980767
01-37 green	449	f	13.9021124

Ps concentrations in Ruffed grouse (continued)

Bird #	Age (days)	Sex	Ps (pmol/mg collagen)
01-23 blue	428	f	14.11459966
01-2 green	457	f	16.78510306
00-22	821	m	27.60021967
98-41	1519	m	51.06680068
01-29 blue	445	m	13.35087993
01-27 blue	421	f	13.46687739
01-20 yellow	442	m	13.14337017
99-22	1173	m	33.3124474
01-37 yellow	430	m	16.37192705
00-34	814	m	28.40609898

Appendix B: Ps Concentrations in Double-crested cormorants

Bird #	Age (months)	sex	Ps (pmol/mg collagen)
878-97644	7	m	3.995
0978-77497	6	m	5.075
878-97738	7	m	5.772
868-94282	7	m	6.032
878-97780	6	m	6.221
0978-76965	6	m	6.325
888-19208	4	m	6.456
878-96952	19	?	10.192
878-10336	5	m	10.762
638-01573	20	f	10.934
878-47605	20	?	11.252
0978-76887	6	?	15.804
789-18655	31	?	15.833
1247-55297	127	m	28.062
1247-28951	137	m	30.827
937-62746	176	m	35.864
2358-15299	162	m	36.759
1227-56154	151	?	38.425
1227-56046	150	m	42.495

Appendix C: Ps concentrations at different sampling locations around body

Bird #	Age (days)	Sex	Body Location	Ps (pmol/mg collagen)
01-28 green	456	m	Breast	17.16805931
			Abdomen	12.22843653
			Thigh	13.8883889
			Lower Back	15.59995709
			Upper Back	14.75206267
			Wing	13.27838059
00-14	823	m	Breast	25.96709472
			Abdomen	23.48110651
			Thigh	24.71840814
			Lower Back	28.32321958
			Upper Back	29.35714804
			Wing	32.34793262
99-22	1173	m	Breast	31.2395585
			Abdomen	29.95866466
			Thigh	43.99255228
			Lower Back	33.00035449
			Upper Back	33.18398572
			Wing	35.50777821
99-18	1174	m	Breast	32.98081229
			Abdomen	27.23330021
			Thigh	56.00490695
			Lower Back	31.6982323
			Upper Back	36.18550146
			Wing	38.1353361
98-35	1524	m	Breast	36.80325661
			Abdomen	37.87481562
			Thigh	49.7166089
			Lower Back	47.57840232
			Upper Back	42.12028764
			Wing	45.87374421
98-1	1563	m	Breast	30.90955642
			Abdomen	34.48299507
			Thigh	44.60750625
			Lower Back	39.18399574
			Upper Back	36.50039751
			Wing	43.62827515

Appendix D: Stability of Ps in Study Skins

Bird	Age (months)	Sex	Age (days)	Ps (pmol/mg collagen)
98-30	48	m	0 (Before Preparation)	33.02055565
			3 Months	32.78458662
			6 Months	34.9598049
			9 Months	35.19847463
			12 months	37.85400854
94-53	96	m	0 (Before Preparation)	72.20836828
			3 Months	66.78366852
			6 Months	68.5326413
			9 Months	80.59465554
			12 months	66.53265444
00-21	24	m	0 (Before Preparation)	19.64351154
			3 Months	22.91367152
			6 Months	22.91367152
			9 Months	25.13508429
			12 months	22.04518206
98-33	48	m	0 (Before Preparation)	31.86523545
			3 Months	33.44893031
			6 Months	34.87625928
			9 Months	40.12600245
			12 months	40.03311143
00-34	24	m	0 (Before Preparation)	28.40609898
			3 Months	26.06809879
			6 Months	27.82159414
			9 Months	26.40104245
			12 months	24.61213105
94-93	96	m	0 (Before Preparation)	64.94534419
			3 Months	73.17937634
			6 Months	59.4208736
			9 Months	67.98261325
			12 months	65.89652443

Literature Cited

Amadori, M. 1931. Condensation products of glucose with p-
     toluidine. Atti della Accademia Nazionale dei Lincei,
     Classe di Scienze Fisiche, Matematiche e Naturali,
     Rendiconti 13:72-77.

Barja, B. 2004. Mammalian and bird aging, oxygen radicals,
     and restricted feeding. Pages 173-189 in Model Systems
     in Aging (T. Nystrom, Ed.) Springer-Verlag Berlin
     Heidelberg, New York.

Baker, G. T., III and R. L. Sprott. 1988. Biomarkers of
     aging. Experimental Gerontology 23:223-239.

Baldwin, S.P., Oberholser, H.C., and L. G. Worley. 1931.
     Measurements of birds. Scientific Publication
     Cleveland Museum of Natural History II.

Baynes, J. W. 1991. Role of oxidative stress in development
     of complications in diabetes. Diabetes 40:405-412.

Bellmunt, M. J. M Portero, R. Pamplona, L. Cosso, P.
Odetti, and J. Prat. 1995. Evidence for the Maillard
reaction in rat lung collagen and its relationship
with solubility and age. Biochimica et Biophysica Acta
1272:53-60.

Beuchat, C.A., and C.R. Chong. 1998. Hyperglycemia and its
consequences for hemoglobin glycation. Comparative
Biochemistry and Physiology. A120:409-416

Bierhaus A., Chevion S., Chevion M., Hofmann M.,
Quehenberger P., Illmer T., Luther T., Berentshtein
E., Tritschler H., Muller M., Wahl P., Ziegler R., P.
and P. Nawroth. 1997. Advanced glycation end product-
induced activation of NF-kappaB is suppressed by
alpha-lipoic acid in cultured endothelial cells.
Diabetes 46:1481-90.

Bucala, R. and A. Cerami. 1992. Advanced glycosylation:
chemistry, biology, and implications for diabetes and
aging. Advances in Pharmacology 23:1-34.

Bunn, H. F., and P.J. Higgins. 1981. Reaction of
     monosaccharides with proteins:possible evolutionary
     significance. Science 213:222-224.

Cerami, A., Vlassara, J., and M. Brownlee. 1987. Glucose
     and aging. Science 256:90-96.

Chaney, R. C., Blemings, K. P., Bonner, J. and H.
     Klandorf. 2003. Pentosidine as a measure of
     chronological age in wild birds. Auk:394-399.

Del Maestro, R. F. 1980. An approach to free radicals in
     medicine and biology. Acta Physiologica Scandinavica,
     Supplementum 492:153-168.

Dyer D. G., J. A. Dunn, S. R. Thorpe, K. E. Bailie, T. J.
     Lyons, D. R. McCance, and J. W. Baynes. 1993.
     Accumulation of Maillard reaction products in skin
     collagen in diabetes and aging. Journal of Clinical
     Investigation 91:2463-2469.

Fayle, S. E. and J.A. Gerrard. 2002. Pages 1-8 in The
     Maillard Reaction (P. S. Beldon, Ed.) Royal Society of
     Chemistry, Great Britain.

Fisher, H.I. 1975. Longevity of the Laysan albatross, *Diomedia immutabius*. Bird Banding 46:1-6.

Greenberg, R. E., S. L. Etter, and W. L. Andersson. 1972. Evaluation of proximal primary feather criteria for aging wild pheasants. Journal Wildlife Management 36:700-705.

Hayase F, R. H. Nagaraj, S. Miyata, F. G. Njoroge, and V. M. Monnier. 1989. Aging of proteins: immunological detection of a glucose-derived pyrrole formed during maillard reaction in vivo. Journal of Biological Chemistry 264:3758-3764.

Heyns, K., Paulsen, H., Eichstedt, R., and M. Rolle. 1957. The formation of 2-aminoaldoses by rearrangement of ketosylamines. Chemische Berichte 90:2039-49.

Holmes, J. D., and S. N Austad. 1995. Birds as animal models for the comparative biology: prospectus. Journal of Gerontology: Biological Science. 50A:B59-B66.

Holmes, D.J., R. Fluckiger, and S.N. Austad. 2001. Comparative biology of aging in birds: an update. Experimental Gerotology 36:849-883.

Ikeda, K., Nagai, R., Sakamoto, T., Sano, H., Araki, T., Sakata, N., Nakayama, H., Yoshida, M., Ueda, S., and S. Horiuchi. 1998. Immunochemical approaches to AGE-structures: characterization of anti-AGE antibodies. Journal of Immunological Methods 215:95-104.

Iqbal, M., Probert, L.L., and H. Klandorf. 1997. Effect of dietary aminoguanidine on tissue pentosidine and reproductive performance in broiler breeder hens. Poultry Science 76:1574-1579.

Iqbal, M., Probert, L.L., Al-humadi, N.H. and H. Klandorf. 1999a. Protein glycosylation and advanced glycosylation endproducts (AGEs): An avian solution. Journal of Gerontology: Biological Science 54:B1-B6.

Iqbal, M., Kenney, P.B., and H. Klandorf. 1999b. Age-related changes in meat tenderness and tissue pentosidine: Effect of diet restriction and

aminoguanidine in broiler breeder hens. Poultry

Science 78:1328-1333.

Johnsen, A., Lifjeld, J. T., and C. Krokene. 2003. Age-
Related Variation in Mate-Guarding Intensity in the
Bluethroat (*Luscinia svecica*). Ethology 109:147— 158.

Klimkiewicz, M. K. and A. G. Futcher. 1989. Longevity
records of North American Birds: Supplement I. J.
Field Ornithology. 60(4):469-494.

Kohn, R. R., Cerami, A., and V. M. Monnier V.M. 1984.
Collagen aging in vitro by non-enzymatic glycosylation
and browning. Diabetes. 33:57-59.

Kohn, R. R., Cerami, A., and V. M. Monnier V.M. 1984.
Collagen aging in vitro by non-enzymatic glycosylation
and browning. Diabetes 33:57-59.

Kun, R. and F. Weygand. 1937. Chemische Berichte 70:769.

Ledl, F. and E. Schleicher 1990. The Maillard reaction in
food and in the human body - new results in chemistry,

biochemistry and medicine. Angewandte Chemie 102:597-626.

Lindstedt, S. L., and W. A. Calde. 1976. Body size and longevity in birds. Condor 78:91-94, 1976.

Maekawa T., Rathinasamy T. K., Altman K. I., and W. F. Forbes. 1970. Changes in collagen with age. The extraction of acid soluble collagens from the skin of mice. Experimental Gerontology 5:177-186.

Maillard, L. C. 1912. Action of amino acids on sugars. Formation of melanoidins in a methodical way. Comptes Rendus de l'Académie des Sciences . 154:66-68.

Makita, Z., Vlassara, H., Rayfield, E., Cartwright, K., Friedman, E., Rodby, R., Cerami, A., and R. Bucala. 1992. Hemoglobin-AGE:a circulating market of advanced glycosylation. Science 258:651-6533.

Maransky, B. P. and K. L. Bildstein. 2002. Follow Your Elders: Age-related Differences in the Migration Behavior of Broad-winged Hawks at Hawk Mountain

Sanctuary, Pennsylvania. The Wilson Bulletin 113:350–353.

Masoro, E. J., M. S. Katz, and C. A. McMahan. 1992. Evidence for the glycation hypothesis of aginag from the food- restricted rodent model. Journal of Gerontology 44:B202-B208.

Masoro, E. J. 1988. Physiological system markers of aging. Experimental Gerontology. 23:391-394.

Monnier V.M, and A. Cerami. 1981. Nonenzymatic browning in vivo: possible process for aging of long-lived proteins. Science 211:491-493.

Monnier V.M., and Cerami A. 1983. Detection of nonenzymatic browining products in the human lens. Biochemica Biophysical Acta. 760:97-103.

Monnier V., M., Kohn R., R., and A. Cerami. 1984. Accelerated age-related browning of human collagen in diabetes mellitus. Proceedings of the National Academy of Sciences of the United States of America 81:583-587.

Monnier, V.M.,R.R. Kohn and A. Cerami. 1994. Accelerated age-related browning of human collagen in diabetes mellitus. Proceedings of the National Academy of Sciences of the United States of America 81:583-587.

Odetti, P., J. Fogarty, D.R. Sell, and V.M. Monnier. 1992. Chromatographic quantitation of plasma and erythrocyte pentosidine in diabetic and uremic subjects. Diabetes 41:153-159.

Payne, R.B. and M.D. Sorenson. 2003. Museum collections as sources of genetic data. Bird Collections in Europe: the Challenge of Mutual Cooperation. Bonner Zoologische Beiträge 51:97-104.

Pickering, S.P.C. 1988. Attendance patterns and behaviour in relation to experience and pair-bond formation in the Wandering Albatross *Diomedea exulans* at South Georgia. *Ibis* 131: 183-195.

Pokharna, H. K., V. M. Monnier, B. Boja, and R. W. Moskowitz. 1995. Lysyl oxidase and Maillard reaction-

mediated crosslinks in aging osteoarthritic rabbit
cartilage. Journal of Orthapedic Research 13:13-21.

Rendell M., Stephen P M., Paulsen R., Valentine J L.,
Rasbold K., Hestorff T., Eastberg S., and D. C. Shint.
1985. An interspecies comparison of normal levels of
glycosylated hemoglobin and glycosylated albumin.
Comparative biochemistry and physiology. B,
Comparative biochemistry 81:819-822.

Sell, D.R. and Monnier, V.M. 1989a. Isolation,
purification, and partial characterization of novel
flurophores from aging human insoluble collagen-rich
tissue. Connective Tissue Research 19:77-92.

Simon, H. and Kraus, A. 1970. Mechanism studies on
glycosylamines, sugar hydrazones, Amadori
rearrangement products and osazones. Fortschritte der
Chemischen Forschung 14:430-471.

Sell, D.R. and Monnier, V.M. 1989b. Structure elucidation
of a senescence cross-link from human extracellular
matrix. Implication of pentoses in the aging process.
Journal of Biological Chemistry 264:21597-21602.

Sell, D. R. and V. M. Monnier. 1990. End-stage renal

    disease and diabetes catalyze the formation of a

    pentose-derived crosslink from aging human collagen.

    Journal of Clinical Investigation 85:380-4

Sell, D.R., A. Lapolla, P. Odetti, J. Fogarty and V. M.

    Monnier. 1992. Pentosidine formation in skin

    correlates with severity of complications in

    individuals with long-standing IDDM. Diabetes 41:1286-

    1291.

Sell, D. R., Lane, M., Johnson, W. A, Masoro, E. J., Mock,

    O. B., Reiser, K. M., Fogarty, J. F., Cutler, R. G.,

    Ingram, D.K., Roth, G. S., and V. M. Monnier. 1996.

    Longevity and the genetic determination of collagen

    glycoxidation kinetics in mammalian senescence.

    Proceedings of the National Academy of Science, USA

    93:485-490.

Sell, D. R., N. R. Kleinman and V. M. Monnier. 2000.

    Longitudinal determination of skin collagen glycation

    and glycoxidation rates predicts early death in

    C57BL/6NNIA mice. The FASEB Journal 14:145-156.

Smith, C. M., Cooke, F., Robertson,G. J., Goudie, R. I.,
and W. S. Boyd. 2000. Long-term pair bonds in
Harlequin ducks. The Condor 102:201-205.

Suzuki, D., Miyata, T., Saotome, N., Horie, K., Inagi, R.,
Yasuda, Y., Uchida, K., Izuhara, Y., Yagame, M.,
Sakai, H., K. Kurokawa. 1999. Immunohistochemical
evidence for an increased oxidative stress and
carbonyl modification of proteins in diabetic
glomerular lesions. Journal of the American Society of
Nephrology. 10:822-832.

Strehler, B. L. 1962. Time, cells and aging. Academic
Press, New York.

Terres, J. K. 1980. The Audubon Society Encyclopedia of
North American Birds. Alfred A. Knopf, New York, NY.

Verzijl., N., Degroot, J., Thorpe, S.R., Bank, R.A., Shaw
J.N., Lyons, T.J., Bijlsma, J.W.J., Lafeber, F.P.J.,
Baynes, J.W., and J. M. TeKoppele. 2000. Effect of
collagen turnover on the accumulation of advanced

glycation end products. Journal of Biological

Chemistry 275:39027-39031.

Winker, K. 2000. Obtaining, preserving, and preparing bird

specimens. Journal of Field Ornithology 71:250-297.