Project Number: MB2-0901

CORRELATING PENTOSIDINE LEVELS WITH AGE IN AVIAN SPECIES

A Major Qualifying Project Report

Submitted to the Faculty

of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in Biochemistry

by

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Jennifer LaPierre

and Biology & Biotechnology

by

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Alyssa Vincent

April 29, 2010

Approved:

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Prof. Michael Buckholt, Major Advisor

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Prof. Jill Rulfs, Co-Advisor

ABSTRACT

A major qualifying project was established last year to create a time and cost effective assay by which to age avian species. It was found that this could be done by measuring and comparing collagen and pentosidine concentrations. This year, the assay was improved upon in regards to time, cost, and overall efficiency. The final assay consists of skin preparation, cell lysis, fluorometry, and collagen determination.

ACKNOWLEDGEMENTS

We would like to thank numerous individuals for making this project successful. First, thank you to Dr. Michael Buckholt and Dr. Jill Rulfs for their constant guidance, support, and valuable resources. Additionally, we would like to thank Dr. Mark Pokras at the Wildlife Clinic at the Cummings School of Veterinary Medicine at Tufts University. Dr. Pokras generously donated all skin samples and the use of the Wildlife Clinic facilities whenever needed. We would also like to thank him for offering his knowledge on necropsy and avian species. We would like to thank Dr. Vincent Monnier of Case Western Reserve University for providing us with a pure stock pentosidine sample. We would like to thank Dr. Destin Heilman for his problem-solving abilities and his assistance with fluorometry. Lastly, we would like to thank those at the Worcester Polytechnic Institute who assisted in the completion of this project.

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INTRODUCTION

GENERAL INFORMATION ON AGING STUDIES

In many species, visual cues are used to help estimate age. Certain characteristics, such as wrinkling of the skin in humans and wear of the teeth and lightening of the muzzle in other mammalian species, have been used as visual markers. Birds, however, have few reliable indicators of age. Throughout evolution, certain anatomical differences in avian species have developed, contributing to the difficulty in reliable aging. The bony jaw and teeth evolved into a beak, and skin evolved feathers to help with flight and thermoregulation (Chaney et al., 2003). The visual markers used in mammalian species are not present in avian species. Scientists can identify changes in plumage, length of tail-feathers, and spur length to determine the point at which some species reach adulthood, but once this stage is reached, visual clues offer no insight into the age of birds (Fallon et al., 2006).

Differences in Anatomical Aging in Avian and Mammalian Species

Aging is defined when the following two criteria are met: 1) characteristic changes in phenotype occur in all individuals over time due to limiting processes and 2) the probability of death at any point increases with the age of the organism. In essence, the concept of aging is an organism's inability to maintain homeostasis (Johnson et al, 1999). Weindruch (2001) proposed several explanations for the aging process, including damage from oxidative stress and glycation reactions, increases in body temperature in association with a hyper-metabolic state, alterations in gene and protein expression, and neuroendocrine changes.

In physiological terms, avian species age at a slower rate than mammals, although variations in aging rates exist within different avian species. The maximum longevity of wild birds is approximately 1.7 times greater than mammals, while the longevity of captive birds is

about three times as long (Austed and Fischer, 1991). However, birds have metabolic rates approximately 2.5 times greater than mammals, an average body temperature 3°C higher, and plasma glucose 2 – 6 times higher than that of mammals. Although these characteristics typically accelerate the aging process, it has been proposed that birds have evolved mechanisms to counteract the negative effects (Iqbal et al, 1999).

With relatively higher metabolic rates, birds are required to consume a greater amount of oxygen per gram of tissue than mammals of comparable body size. This produces reactive oxygen species, which are known to contribute to cellular damage, which is associated with aging. Holmes et al. (2001) proposed that birds have developed two mechanisms to minimize oxidative damage. Birds may have an increased antioxidant activity, which could potentially provide resistance to damage from the reactive oxygen species. They may also produce less reactive oxygen species than mammals, which would directly decrease cellular damage. Similar theories have been proposed to explain the greater longevity in avian species, despite higher metabolism, body temperature, and blood glucose.

Importance of knowing ages in bird studies

A defined procedure for estimating the age of avian species could greatly benefit aviculture, ornithology, and science in general. Knowledge regarding the longevity of avian species could provide information for species survival programs, rescue efforts, and scientific advancements (Chaney et al., 2003). The results of bird studies could provide valuable information regarding lifecycles and population fluxes, including maximum and average life span, generational span, and onset and length of reproductive cycle (Fallon et al., 2006).

Such information could have a significant effect not only on the database of general avian knowledge, but on the survival of the most critically endangered species as well. Reliable age

estimation could directly impact species survival plans and the pairing of endangered species. Genetic compatibility and similarity in age are extremely important when pairing endangered species, since many birds mate for life. The ability to estimate age in wild birds could significantly increase compatibility, ensuring the production of best-fit offspring. Zoo programs, which are involved in small population management, would also benefit in terms of compatibility when mating, and research in terms of behavioral patterns and species-specific physiology (Chaney et al., 2003).

Wildlife clinics, like the wildlife clinic at the Tufts Center for Conservation Medicine, would greatly benefit from the ability to age avian species. The Tufts Wildlife Clinic provides medical care for orphaned, sick, and injured New England wildlife. This includes a variety of native amphibians, reptiles, birds, and mammals. The animals are brought to the clinic by citizens, local wildlife rehabilitators, and regional, state, and federal fish and wildlife agencies. At the clinic, the animals are assessed and treated accordingly (Tufts University, 2006). If approximate age estimation was possible at the clinic, the medical staff would be able to treat avian species with a better knowledge of possible recovery and release back into the wild.

During an interview on January 21, 2010, Mark Pokras, an associate professor in the Department of Environmental and Population Health, as well as Wildlife Medicine, commented on the limitations that wildlife clinics currently face and the vast implications associated with estimating age. He stated that the lack of a known or approximate age of a bird "forces you back a step in terms of diagnostic medicine. Aging an animal allows you to use your time and resources wisely" (M. Pokras, personal communication, January 21, 2010). Tufts Wildlife Clinic mainly sees trauma victims. Since the chances of healing differ with age, this factor can be used in triage when determining chance of survival and allocating medical resources. Estimating the

age of birds can also open doors to different areas of scientific research, such as studies in toxicology and non-contaminant disease. When studying the accumulation of chemicals or incidence of fungal respiratory disease, "age is the one variable we have no handle on in a bird's life" (M. Pokras, personal communication, January 21, 2010). The implications of aging birds are far reaching both in wildlife medicine and preservation.

Common Loon (*Gavia immer)*

The Common Loon *(Gavia immer)* is of the family gaviidae and order of gaviiformes. The average life span of the Common Loon is 30 years (National Geographic Society, 2009). The Common Loon is a large water bird weighing an average of 2500 – 6100 g, with a long body that slopes to the rear and sits low on the water (Cornell Lab of Ornithology, 2009). Common Loons have red eyes, a black head and neck, white stripes, and checkered and spotted patterns on their backs (National Geographic Society, 2009). The average wingspan is 104 – 131 cm and the average length is 66 – 91 cm (Cornell Lab of Ornithology, 2009). Juvenile Common Loons have a uniform edge between their underside and upper side, the feathers on their back have a scalded edge of white on the feathers, and their bills are slightly paler than mature loons (Cornell Lab of Ornithology, 2009). The average clutch size is 2 to 4 eggs, which are brown with dark splotches (Cornell Lab of Ornithology, 2009). The nest usually consists of a mass of plant matter and is sometimes found on the top of muskrat dirt mounds (Cornell Lab of Ornithology, 2009). Hatchlings leave the nest one day after hatching and can fly in 11 weeks (National Geographic Society, 2009).

Common Loons are water birds, and only go on land to mate and nest (Cornell Lab of Ornithology, 2009). Their normal migration pattern is to breed on fresh water lakes with rocky shorelines and forest, stage for migration on lakes and rivers, and winter primarily on coastal

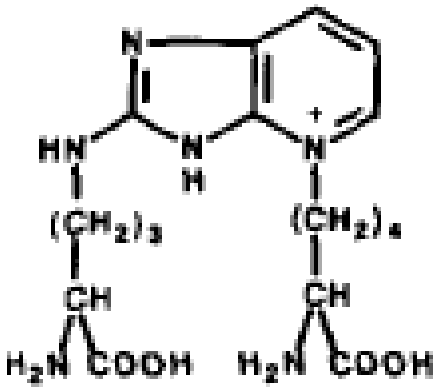
marine areas near shore (Cornell Lab of Ornithology, 2009). Common Loons normally winter along the North America Pacific and Atlantic coasts (National Geographic Society, 2009).

Loons are carnivores, with a diet consisting mostly of fish and some aquatic vertebrates and invertebrates (Cornell Lab of Ornithology, 2009). The status of the Common Loon in the IUCN database is in the category of "least concern," or "currently stable". In the early 20th century, the Common Loon population decreased, but in the late 20th century, populations increased to stable levels (Cornell Lab of Ornithology, 2009). Common Loons have been on endangered species lists in many northeastern United States (Sidor et al., 2003). The most studied and common threats to Common Loon populations are toxicosis, contamination from oil spills, respiratory diseases such as aspergillosis, botulism, trauma, entanglement in fish line or nets, and emaciation of unknown origin (Sidor et. al, 2003). The major cause of death in chicks is aggression from other species and trauma (Sidor et al., 2003). The major cause of death in immature loons is fungal infections of the respiratory tract and trauma (Sidor et al., 2003). Adults that are wintering die most often from trauma and infection while breeding adults die often from lead toxicosis (Sidor et al., 2003). Related species include the Red-Throated Loon (*Gavial stellata),* Pacific Loon (*Gavial pacifica),* Yellow-billed Loon, and Cormorant (Cornell Lab of Ornithology 2009).

PENTOSIDINE

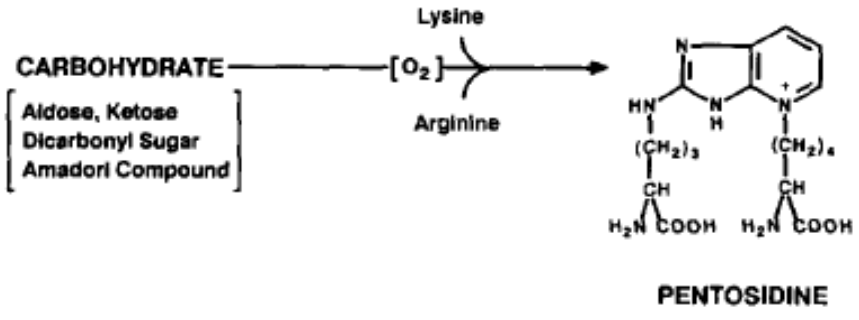
Pentosidine is a fluorescent protein cross-link that is formed by non-enzymatic glycation of protein and sugars in collagen (Chaney et al., 2003). Specifically, pentosidine consists of a lysine and arginine residue and a pentose molecule (Chaney et al., 2003).

Figure 1: Pentosidine Structure (Dyer, 1991)



Pentosidine is found in collagen in lung, aortic, kidney, tracheal, cardiac, dura, lens, fibroblast, and glomerular mesangial tissue, and accumulates irreversibly over the lifespan of an individual (Chaney et al., 2003). It can therefore be used as a biological marker in aging studies. Pentosidine was discovered in 1989 in the outer layer of the membranes surrounding the brain and spinal cord or dura matter of humans (Sell and Monnier, 1989a). Pentosidine has now been researched thoroughly in humans and many other species. There are three pathways in which pentosidine can be synthesized in the body. All three pathways follow the basic scheme depicted in Figure 2.

Figure 2: Basic Scheme of Pentosidine Formation (Dyer, 1991)



AGE Introduction and Accumulation

Pentosidine is a member of a group known as Advanced Glycation Endproducts, or AGEs. AGEs are formed by the Maillard Reaction, which involves reacting an amino acid and a reducing sugar (Chaney et al., 2003). Accumulation of AGEs alters protein structure, such as collagen in this case, and reduces these proteins' susceptibility to catabolism (Dyer, 1991). AGEs cause yellowing and cross-linking in proteins considered "long lived," including collagen and lens crystalline (Chaney et al., 2001).

Many aspects of an individual's life can affect AGE accumulation. It has been found that diet and upbringing in captivity or in the wild can affect AGE accumulation (Fallon, 2003). Restrictive diets, such as the need to forage, decrease AGE accumulation (Fallon, 2003). However, the exercise needed to forage can lead to oxidative damage and an increased AGE concentration (Fallon, 2003). AGE accumulation has not been found to differ significantly between sexes in avian species (Fallon, 2006). Because metabolic rate is inversely related to AGE accumulation, birds with longer life spans tend to have a lower AGE accumulation (Chaney et al., 2003).

Many other differences can be speculated, but have not been thoroughly explored. However, it is clear that there will most likely never be a generic pentosidine/aging curve that will hold relevant to all avian species. It is reasonable to assume that overlap between many species could be possible. For example, due to similar environment, diet, and lifespan, it would be likely that Red-Throated Loon, Pacific Loon, Yellow-billed Loon, and Cormorant would have similar pentosidine/aging curves.

Collagen

Collagen is a long rod-like connective tissue that constitutes approximately 30% of protein in mammals (Chaney et al., 2001). This tissue is a non-renewable structure and therefore changes drastically with age (Kohn and Schnider, 1982). Structural changes occur, which are caused by a lack of elasticity (Kohn and Schnider, 1982). The collagen itself becomes less susceptible to collagenase and heat denaturation, and more insoluble (Kohn and Schnider, 1982).

There are three major types of collagen: Type I, which forms connective tissue between skin, bones, tendons, ligaments, dentine, fascia, and organ capsules; Type II, which forms cartilage, notochord, and vertebrate disks; and Type III, which forms connective tissues of organs, smooth muscle, endoneunum, blood vessels, and fetal skin (Chaney et al., 2001). However, there are 19 unique forms of collagen, each with distinct functions within the body (Chaney et al., 2001).

Skin collagen has an accepted half life of 15 years in mammals (Chaney et al., 2001). As previously stated, with the accumulations of advanced glycation endproducts and crosslinking, collagen increases in rigidity and tension (Chancey et al., 2001). Crosslinking occurs by two different methods. First, it can be enzymatically catalyzed by the enzyme lysyl oxidase (Chaney et al., 2001). This is a normal process instigated by maturation processes (Chaney et al., 2001). The second method is nonenzymatic and involves carbohydrate linkage (Chaney 2001). This process, along with stress, causes accelerated maturation (Chaney et al., 2001). The causes of the nonenzymatic crosslinking process are not completely understood. It is generally accepted that nonenzymatic crosslinking is caused by oxidative stress and is related to low metabolic rate,

temperature, vascularization, and collagen turnover. Pentosidine is formed by this non-enzymatic process (Fallon, 2006).

Localization of Pentosidine in Avian Tissues

Pentosidine levels have been found to vary in different areas of the body in the same subject (Fallon, 2006). This is due to differences in vascularization, tissue temperature, collagen turnover rates, and antioxidant concentration (Fallon, 2006). These differences cause the collagen to be exposed to different levels of oxidative stress, thereby accumulating pentosidine differently in different tissues (Holmes, 2001).

Research has demonstrated that pentosidine levels are highest in the thigh, wing, and breast (Fallon, 2006). In theory, the webbing of the foot would be the easiest area of the body of the Common Loon to perform a punch biopsy (Chaney et al., 2003). However, pentosidine has been found to be at the lowest concentration here (Chaney et al., 2003). This is caused by the low vascularization of the webbing, the fact that the webbing is at a consistently lower temperature than the rest of the body, which slows the non-enzymatic process, and the high concentration of uric acid present (Chaney et al., 2003). Uric acid is an antioxidant that prevents oxidative damage and therefore cross-linking accumulation (Chaney et al., 2003). Pentosidine is related to oxidative stress because oxygen is required for its synthesis (Fallon, 2006).

Pentosidine in Birds as a Marker of Age

Numerous studies have been conducted to demonstrate that pentosidine is a reliable biomarker that can be used to age birds (Iqbal et al., 1999; Chaney et al., 2003; Fallon et al., 2006). Since this compound is present in the collagen found in the skin of birds, research on the use of pentosidine to accurately estimate age has been conducted.

PREVIOUS METHODS AND RESULTS

The first major breakthrough in research concerning the use of pentosidine as a biomarker for avian age occurred in 1999, when Iqbal *et al*. confirmed that pentosidine is present in the skin of broiler hens (*Gallus neglectedus*), and its concentration increases with age. In the study, hens were randomly selected and euthanized. Approximately 1g of skin was removed from the breast and the samples were prepared by removing the epidermal and adipose layers from the skin samples, freezing in liquid nitrogen, mincing, delipidation, rehydration, and hydrolyzation. Estimation of pentosidine levels was then conducted using a modified reversed phase HPLC method. Results indicated an age-related, linear increase in pentosidine levels.

In 2003, Chaney *et al*. conducted studies to determine the relationship between pentosidine accumulation and age, and the possible differences due to the location in which the skin samples were obtained. Forty five birds of 29 varying species were obtained, with known ages ranging from a few days to 18.5 years. Skin samples were obtained from the thorax and abdomen. In a second study, skin samples from the foot webbing of 17 California Gulls (*Larus callifornicus*) were obtained. A similar method to that used by Iqbal et al. (1999) was employed. Pentosidine was found in the skin of various species of wild birds, and its concentration increased linearly with age within species in both skin from the breast and the foot webbing. The mean collagen content in the breast skin and foot webbing was not significantly different, but the pentosidine concentration was higher in breast skin than in foot webbing.

In 2006, Fallon *et al.* conducted a study to determine potential differences in pentosidine accumulation in different avian species. Pentosidine accumulation in the skin of Ruffed Grouse (*Bonasa umbellus*) and Double-Crested Cormorants (*Phalacrocorax auritus*) was compared. Skin samples were obtained from the breasts of 52 Ruffed Grouse of known age, ranging from a

few days to 10 years, and 19 Double-crested Cormorants. The skin samples were prepared using a method similar to Iqbal et al. (1999) and Chaney et al. (2003). However, pentosidine concentration was measured using high-performance liquid chromatography with an in-line fluorescence detector. Pentosidine concentration increased with age in both species of birds, with no significant difference due to sex. A more rapid increase in pentosidine concentration was found in the Ruffed Grouse than in the Cormorants. It was then concluded that pentosidine calibration would need to be conducted for individual species, as no generalized avian curve could be created.

In a second study conducted by Fallon et al. (2006), the effect of time on the stability of pentosidine concentration, as well as the variation in pentosidine concentration among varying locations of the body, were investigated. In the first part of the study, skin samples were obtained from six male Ruffed Grouse of known ages, treated with hydrous sodium borate, and stored at -80°C until needed. The skins were then sewn onto cotton bodies and allowed to air dry in a well-ventilated location, rotating position every third day to ensure even drying. Samples were removed from the cotton bodies every three months over the course of one year to test for pentosidine concentration over time. The samples were then prepared according to the methods used by Iqbal et al. (1999) and Chaney et al. (2003). It was determined that pentosidine concentrations remained stable over the course of one year in the six Ruffed Grouse. This provided evidence that pentosidine accumulation can be used to estimate age of birds that have been properly prepared and preserved as study skins.

In the second part of the study, skin samples were obtained from the breast, abdomen, lower back, upper back, right wing, and right thigh of six male Ruffed Grouse of known age. Samples were taken at necropsy right after death and prepared according to the methods used by

Iqbal et al. (1999) and Chaney et al. (2003). It was found that pentosidine concentrations in skin collagen differ among various locations on the body. Skin samples taken from the thigh and wings had significantly higher concentrations than those from the breast. Pentosidine concentrations in the abdomen and upper and lower back did not differ significantly from those in the breast.

GOALS OF THE PROJECT

The goal of this work was to establish the criteria for an assay to determine pentosidine concentration in the collagen of the Common Loon. From this, a pentosidine aging curve would be established to assist in the aging of both banded and non-banded Common Loons.

BASIC EXPERIMENTAL PROCEDURE

The final assay is comprised of the following steps: skin preparation, cell lysis, fluorometry, and collagen determination. First, a skin sample is defeathered using paraffin wax. The sample is then minced into small sections and blended to make the following extractions more efficient. A double extraction method is employed using RIPA buffer to lyse the cells within the sample. The resulting supernatant is collected and the emission of the pentosidine present is determined using fluorometry. A standard curve is created with a sample of known pentosidine concentration, which allows for the concentration of pentosidine in the samples to be determined. The following step, collagen determination, has been unsuccessful due to difficulty in producinga standard curve. Theoretically, however, the collagen concentration could be determined spectrophotometrically with the use of Direct Red 80 dye to label collagen. Total collagen determination must be performed in order to standardize the pentosidine readings. From this, it would be possible to determine the concentration of pentosidine present in a fixed amount of collagen. A Bradford Assay and an altered Bradford Assay were conducted to

measure total protein in the sample, for use as a potential alternative to collagen in normalizing pentosidine. After determining the concentration of pentosidine and collagen, the ratio of pentosidine to collagen could be determined. Theoretically, the higher this ratio, the older the bird the sample was obtained from.

GENERAL SPECIMEN COLLECTION

For the purposes of this work, the left breast tissue was used. This is not an ideal location for collection because the underlying muscle is involved in flight. In the future, if the assay was perfected and could realistically be used on live birds, it may become sensitive enough to make use of the punch biopsy in toe webbing possible. The punch biopsy has been used in past research through the patagium of deceased birds and it has been suggested that this would be the best method for use on live birds (Cooey, 2008). The breast skin is more sensitive in regards to flight function because it covers important muscle groups (Cooey, 2008). In Cooey *et al*'s study it was recommended that the best skin harvesting procedure involves taking a $6mm^2$ sample from the patagium and then closing the wounds with tissue glue.

Because multiple banding programs have included punch banding techniques through wing patagium with no adverse effects caused to the life of the birds, it is reasonable to assume that a similar punch biopsy for skin sample purposes would be acceptable (Cooey, 2008). Other advantages to obtaining samples from the patagium include the fact that there are fewer muscles in the wing and the veins are visible, allowing for skin collection without compromising the blood stream (Cooey, 2008). In the following study, only deceased birds were used and therefore, the left breast tissue was most economical to collect to give the largest concentration of pentosidine per gram of tissue.

ASSAY FOR COLLAGEN DETERMINATION

In this assay, both pentosidine and collagen concentrations were measured, and then a ratio was determined. High performance liquid chromatography, known as HPLC, is a widely used method for collagen determination. In this procedure, acid hydrolysis is used for hydroxyproline determination. The collagen content would then be estimated from the hydroxyproline content, using the following equation:

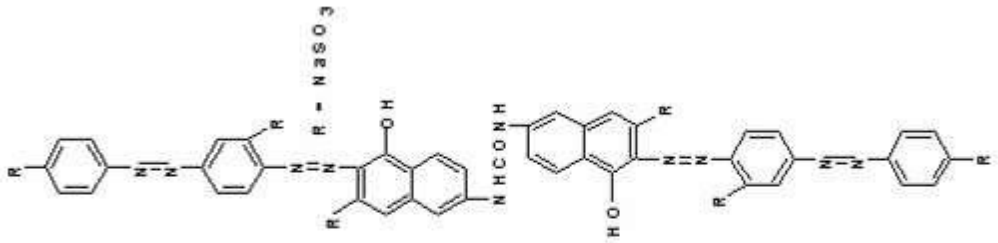
$$\frac{Micrograms\ hydroxyproline\ in\ 1mL\ hydrolysate}{Micrograms\ tissue\ represented\ in\ 1\ mL\ hydrolysate} \times 7.46 \times 100$$

This method can determine collagen concentration in small amounts of tissue with accuracy better than ∓ 5 percent (Pataridis, 2009). However, it involves the use of hydrochloric acid at high temperatures for extended periods of time, dry nitrogen gas, and complex procedures involving instruments unavailable to a clinical lab (Avery et al., 1996). This would prove to be impractical and costly if routinely performed in a clinical lab.

An alternative, practical method for collagen determination was explored. This method, based on the Sircol Soluble Collagen Assay (Biocolor Ltd., 2007), employs cell lysis using a RIPA buffer, followed by the use of Direct Red 80 dye to determine collagen concentration spectrophotometrically. Due to ease of use, as well as cost and utilization of instruments found in clinical labs, this method proved to be the most beneficial for use in a clinical lab setting.

Direct Red 80 dye, also known as Sirius Red, is a strong anionic dye that stains collagen fibers. As seen in Figure 3 below, the dye contains sulfonic acid side chain groups. These groups interact with the basic side groups present in the collagen (Taskiran et al, 1999).

Figure 3: Molecular Structure of Sirius Red



The elongated dye molecules align in a parallel form to the long thread-like structure of the collagen, resulting in a high affinity of the dye for the collagen. Sirius Red has been used in numerous experiments in order to localize collagen within tissues.

The dye has been used along with the extraction of soluble collagen from tissue, cartilage, and organs from various species with results similar to that derived using HPLC (Biocolor Ltd., 2007). For example, Aramwit *et al.* (2009) used the Sircol method with Sirius Red to extract and determine collagen concentration from fibroblast cells, while Banerjee *et al.* (2009) used the method and dye to extract and determine collagen concentration from murine lung tissue. Previous use of Sirius Red suggests that an assay utilizing this dye in the collagen determination step should work.

PROCEDURES

SKIN PREPARATION

Twenty one loon skin samples were obtained from the Wildlife Clinic at Cummings School of Veterinary Medicine at Tufts University during necropsy under the supervision of Dr. Mark Pokras. Samples measuring 3 in x 15 in and weighing approximately 15 g were removed from the left breast of the deceased birds. The skins were immediately stored in a 0.9% NaCl saline solution and placed in a freezer at -20°C until needed. Before the primary experiments could be conducted, the skin samples were defeathered and blended. Several methods were attempted before a final procedure was developed.

Scalding and Detergent

The most common method of feather removal involves hot water scalding. This method was attempted using skin from a Mallard duck (*Anas platyrhynchos*) according to the procedure established by Drs. Roger Jacobs and Don Sloan of the University of Florida (2003). The skin sample was submerged in a hot water bath at a temperature between 71°C and 76°C for approximately 30 seconds. The feathers were then plucked using tweezers. Hot scalding was also attempted with the addition of a tablespoon of Dawn dishwashing detergent to the water bath. It was hypothesized that the detergent would reduce the hydrophobicity of the feathers, causing them to become more susceptible to the hot water scalding. The sample was submerged in a hot water bath containing detergent at a temperature between 71°C and 76°C for 30 seconds. A scalpel, with the blade moving parallel to the feather shafts, was scraped along the skin to try to remove the remaining feathers.

Use of Paraffin Wax

The scalding method above was modified to include a final step using paraffin wax to remove the undercoat and feather shafts. A sample was hot scalded with detergent at a temperature between 71°C and 76°C for a total of 35 seconds, and the feathers were removed with intermittent hot scalding about every 10 seconds. The sample was then dipped in paraffin wax, placed in a beaker of cold tap water for 5 seconds to solidify the wax, and the feathers were removed with tweezers. This was repeated two to three times, until all feathers and shafts were removed.

Feather removal was also attempted without the use of scalding. A sample was alternately dipped in paraffin wax and a beaker of cold tap water two to three times, with manual plucking and tweezing between each dip. The feathers clumped together due to the wax, and both feathers and wax were removed sections at a time. Any remaining feather shafts were removed with the scalpel.

Mincing and Blending

In order to prepare the samples for delipidation, the skin samples were minced using a 10 blade scalpel. The small pieces were then submerged in liquid nitrogen and blended using a Waring blender with an MC2 (37 – 110mL) container. It was important to determine the correct level of liquid nitrogen in the blender to use and the amount of time the samples should remain immersed. Enough liquid nitrogen was added to completely submerge the samples for no more than 10 sec. When the correct amount of liquid nitrogen was used, the skin samples were blended into a powder. This powder was then placed in 15 mL conical tubes and frozen at -80°C until they could be delipidated. To determine if the liquid nitrogen step was necessary, the blending was also performed without it.

DELIPIDATION

The blended skin samples were delipidated in accordance with the procedure provided by Alan and Belen (2009). Two 1.0 g portions of the same defeathered, minced, and blended skin sample were placed in separate 15 mL conical tubes in preparation for a double extraction. To these two tubes, 2.66 mL of chloroform and 1.33 mL of methanol were added. The samples were then mixed using a Mistral Multimixer for 18 to 22 hours. The samples were centrifuged in a Beckman Allegra 6KR centrifuge at 2250 rpm for 10 minutes and the supernatant was removed, leaving the delipidated skin. The skin sample was transferred to new conical tubes.

The necessity of delipidation was explored. Originally, the delipidation step was added to the protocol because lipids are known to fluoresce, and therefore would overlap and distort the fluorescence wavelength of pentosidine present in the sample. Both delipidated and nondelipidated skin cells from the same sample were lysed. Using a Perkin Elmer LS 55 Fluorescence Spectrophotometer set at an emission wavelength of 422 nm and FL WinLab software, the fluoresence values for the delipidated and nondelipidated samples were measured.

CELL LYSIS

After delipidation, the cells of the skin samples were lysed using a double lysing system with RIPA buffer containing the following: 1.0% Nonidet-P40, 0.2% SDS, and 49.5mL of 10mM TRIS buffer at pH 7.4. Lysing allowed for the release of the collagen and pentosidine from the cells, and therefore quantification in following experiments. Four milliliters of RIPA buffer were added to the first of the two 1.0 gram of the same delipidated sample. This was vortexed, allowed to sit at room temperature for 1 hour, and centrifuged in a Beckman Allegra 6KR centrifuge for 10 minutes at 2250 rpm. The supernatant was removed and transferred to a second 1.0 g of the delipidated sample from the same tissue. This was then vortexed, allowed to

sit at room temperature for 1 hour, and centrifuged for 10 minutes at 2250 rpm. The supernatant was then transferred to a new 15 mL conical tube.

Concentration Series using the RIPA Buffer

In order to optimize subsequent collagen pellet formation, the concentrations of detergents in the RIPA buffer were increased systematically. The original concentrations of the components of the RIPA buffer used were as follows: 1.0% Nonidet-P40, 0.2% of SDS, and 49.5mL of 10mM TRIS buffer at pH 7.4. The concentrations of the Nonidet and SDS were increased and corresponding pentosidine levels were measured to determine optimum Nonidet and SDS concentrations. It was assumed that the greater the pentosidine reading, the more effective the extraction of collagen, resulting in a higher collagen concentration and therefore increased ease of precipitation.

FLUOROMETRY

Procedure for Using Fluorometer

The optimal emission wavelength for pentosidine was determined to be 422 nm. A Perkin Elmer LS 55 Fluorescence Spectrometer with FL WinLab software was used to determine the fluorescence values for all samples.

Pentosidine Standard Curve

In order to establish a standard fluorometric curve for pentosidine, 0.5 uM pure pentosidine was obtained from Dr. Vincent Monnier of Case Western Reserve University. The sample was reconstituted in 100 uL of distilled water, creating a 1.89 mg/mL solution of pentosidine (Alan & Belen, 2009). The standard curve was produced using a series of two-fold dilutions. The final concentrations used were 0.0900 mg/mL, 0.0450 mg/mL, 0.0225 mg/mL, 0.0113 mg/mL, 0.0056 mg/mL, 0.0028 mg/mL, 0.0014 mg/mL, and 7.0313×10^{-4} mg/mL.

COLLAGEN ASSAY

The amount of collagen in each sample was to be determined using a method derived from the Sircol Soluble Collagen Assay (Biocolor Ltd., 2007). The necessary dye and alkali reagents were created as instructed in the manual. A concentration of 10 mg/mL Direct Red 80 dye was produced by solubilizing 10 mg of powdered dye in 1 mL of distilled water. A stock concentration of 0.5 M NaOH was used as the alkali reagent.

In accordance with the assay determined by Biocolor Ltd. (2007), 100 uL of each sample was placed in a labeled microfuge tube with 1 mL Direct Red 80 dye. The tubes were placed in a Mistral Multi-mixer mechanical shaker for two hours and then spun in an Eppendorf 5415D microcentrifuge at 13,200 rpm for 15 minutes. The resulting supernatant was removed by gentle inverting. Excess dye was removed using a Kimwipe. A cotton swab was then used in place of a Kimwipe to allow more precise placement of the cotton to ensure that the pellet remained intact. The pellet was then vortexed back into solution with the addition of 1 mL of the 0.5 NaOH alkali reagent. The solution was placed in semi-microcuvettes and the absorbance was read at A_{540} using a Jenway Spectrophotometer. Distilled water was used as a blank.

Removal of Excess Dye

After the sample had spun for 15 minutes in the centrifuge, the supernatant was removed by gentle inverting. Some liquid adhered to the sides of the microfuge tubes and the top of the pellet. A Kimwipe was rolled to a point and dipped into the tube to try to remove the liquid on adhered to the sides. However, it was not possible to remove the liquid above the pellet without disrupting it. As a result, a washing step was added.

Washing Step

In order to obtain more accurate collagen concentrations, a washing step was added to the procedure after the initial removal of the dye by inversion and swabbing. This step allowed for the removal of all excess dye from the microfuge tubes. The pellet was washed using approximately 500 uL of 99% pure, methanol-free ethanol. The ethanol was removed from the microfuge tube by careful inversion so as not to dislodge the collagen pellet. After this step was performed, several cotton swabs were used to absorb the excess liquid. The tip of the swabs allowed for greater control when swabbing close to the pellet. Any disruption in the pellet could have altered the collagen readings.

Collagen Standard Curve

Type I rat tail collagen (Sigma Aldrich cat #C8897), bovine achilles tendon collagen (Sigma Aldrich cat #C9879), and a type I collagen solution from calf skin (Sigma Aldrich cat# C8919) from Sigma were used in an attempt to develop a collagen standard curve. A total of 5 mg of Type I rat tail collagen were solubilized in 5 mL of 0.5 M acetic acid. The final concentrations used were 1000 ug/mL, 200 ug/mL, 100 ug/mL, 40 ug/mL, 20 ug/mL, and 8 ug/mL. This was repeated with the bovine achilles tendon collagen. As a last attempt, type I collagen solution from calf skin was purchased from Sigma and used to create a standard curve. One sample was prepared using 100 uL of the collagen solution in 1 mL of Direct Red 80 dye to determine if a collagen pellet would form. Collagen samples from various sources were used due to a failure to form a collagen pellet.

BRADFORD ASSAY

Due to the inability to create a collagen standard curve using the Direct Red 80 dye protocol, another approach was attempted. Instead of measuring collagen concentration specifically, a Bradford

Assay was used in order to measure total protein concentration in samples. The total protein concentration would include collagen. A protein standard curve was created to evaluate our samples using stock solution of 2 mg/mL Bovine Serum Albumin (cat # 23209) purchased from Piece, and a serial dilution was performed. 0.05mL of each dilution was added to 1.5 mL of Coomassie Plus Reagent and allowed to incubate for 10 minutes at room temperature. The samples were transferred to microcuvettes and the absorbance values read at 595nm in a Jenway Spectrophotometer. Distilled water was used to calibrate the spectrophotometer. The same procedure was used for each skin sample and the standard curve was used to determine the estimated protein concentration.

ALTERED BRADFORD ASSAY

An altered Bradford Assay was performed in order to increase the Coomassie Plus Reagent's affinity for collagen and minimize the reagent's affinity for other proteins in solution (Duhamel 1981). It has been determined that by adding a small amount of SDS, around 0.0035%, to the Coomassie Plus Reagent, the amount of collagen measured would increase by a magnitude of one and the amount of non-collagen proteins measured would decrease by a magnitude of two (Dunhamel 1981). Fifteen microliters of 10% SDS solution were added to 50 mL of Coomassie Plus Reagent (Lopez 1993). Using a stock solution of 1 mg/mL type I collagen solution from calf skin, a serial dilution was performed. 0.05mL of each dilution was combined with 1.5mL of the SDS/Coomasie Plus Reagent combination. These combinations were allowed to incubate for 10 minutes at room temperature. The samples were transferred to microcuvettes and the absorbance values read at 595nm in a Jenway Spectrophotometer. Distilled water was used to calibrate the spectrophotometer. The same procedure was used for each skin sample and the standard curve was used to determine the estimated collagen concentration.

FINAL ASSAY

The samples were prepared using paraffin wax during the defeathering process, mincing with a scapel blade, and blending using a Waring Blender. The cells were lysed using RIPA

buffer containing the following: 1.0% Nonidet-P40, 0.2% SDS, and 49.5mL of 10mM TRIS buffer at pH 7.4. Four milliliters of RIPA buffer were added to l g of skin sample, vortexed, and allowed to sit for 1 hour. The supernatant was extracted after centrifugation and transferred to a conical tube containing a second gram of skin from the same sample. The process was then repeated and the supernatant collected. The emission of pentosidine was then determined using fluorometry. Using a standard curve created with a sample of known pentosidine concentration, the concentration of the pentosidine in the unknown samples was determined. Collagen was then extracted using 100 uL of sample and 1 mL of Direct Red 80 dye. This was mixed for two hours and centrifuged. The precipitated pellet was isolated using Kimwipes and cotton swabs and resuspended into solution using 0.5M NaOH. The absorbance was read using a Jenway Spectrophotomer set to a wavelength of 540nm. However, a collagen standard curve could not be created, so the collagen determination step could not be completed. Since collagen determination via the modified Sircol assay was unsuccessful, an altered Bradford Assay was employed to estimate total protein concentration.

RESULTS & DISCUSSION

GENERAL RESULTS

The original hypothesis stated that the concentration of pentosidine increases in collagen with increasing age in the Common Loon. A pentosidine standard curve was created using fluorometry results. A trendline was determined and used to calculate the concentration of pentosidine in 21 loon skin samples. The final results obtained negated this hypothesis, as it appeared that age and pentosidine concentration were negatively correlated. The collagen assay using the Direct Red 80 dye could not be completed successfully since a standard curve could not be produced. This occurred due to lack of solubilization of collagen and absorbance readings. It is possible that if this assay were to be completed successfully, then the originally hypothesized correlation would be supported. In an attempt to solve this issue and complete the standard curve, a Bradford Assay and an altered Bradford Assay were used to indirectly measure collagen concentration. However, the results were not consistent with the hypothesis as it appeared that the pentosidine to collagen ratio and avian age were negatively correlated.

SKIN PREPARATION

The final skin preparation consisted of plucking using paraffin wax, followed by mincing and blending. It was determined that scalding was not required.

Scalding and Detergent

When the skin sample from the Mallard duck was submerged in a hot water bath containing Dawn dishwashing detergent, the skin fell apart when plucked. This was possibly due to the large amount of adipose tissue in the sample. When another sample was submerged for 25 seconds, the feathers were easily plucked. The scalding and detergent were useful in removing the outer feathers, but proved unsuccessful in the removal of the undercoat and feather shafts.

To try to remove the remaining feathers, a scalpel, with the blade moving parallel to the feather shafts, was scraped along the skin. This was only mildly successful.

The scalding and detergent procedure was then conducted on skin samples of the Common Loon (*Gavia immer)* rather than the skin of the Mallard duck. It was determined that loon feathers were more difficult to remove than the feathers of the Mallard duck. The outer feathers were only partially removed, while the undercoat and feather shafts remained. The sample was submerged several times and the scalpel was used to try to remove the remaining feather shafts, but this method proved to be extremely time-consuming.

Use of Paraffin Wax

The addition of the paraffin wax decreased the time it took to remove the undercoat and feather shafts. When the wax hardened and was removed with tweezers, large sections of feathers detached at a time, which greatly sped up the process. When the paraffin was applied without hot scalding, this method proved to be equally effective as the previous method, but involved less time and materials. As a result, it was determined to be the most successful defeathering method and was employed for the remaining loon skin samples.

Mincing and Blending

When the samples were blended with enough liquid nitrogen to submerge the sample in the blender, the skin was blended into a powdery consistency. However, without the use of liquid nitrogen, the samples became paste-like. When the paste-like samples were run through the entirety of the assay, the consistency did not affect the results. Therefore, the liquid nitrogen was deemed unnecessary and all subsequent samples were prepared without it.

DELIPIDATION

To determine if the delipidation step was necessary, the assay, either with or without the delipidation step, was conducted with several samples. The fluorescence values were then measured and the delipidated and non-delipidated portions from each skin sample were compared. The resulting values are located in Table 1.

Table 1: Pentosidine Fluorescence Values for Delipidated and Non-Delipidated Samples

Tufts Code	Treatment	Fluoresence Value (nm)
07-445	Delipidated	19.122
	Non-Delipidated	21.409
08-169	Delipidated	46.509
	Non-Delipidated	48.459
09-036	Delipidated	16.753
	Non-Delipidated	13.288
09-042	Delipidated	7.065
	Non-Delipidated	20.932
09-093	Delipidated	23.720
	Non-Delipidated	4.453

The fluorescence values for the delipidated samples were not consistently higher or lower than the non-delipidated samples. This indicates that the delipidation process itself is not affecting the emission readings; rather, the differences in wavelengths are most likely due to the collagen differences between the two sections of skin. As a result, it was determined that the delipidation step could be completely removed from the assay, reducing the time the assay takes to complete by almost 24 hours.

CELL LYSIS

The concentrations of Nonidet and SDS were increased systematically in order to determine the concentration at which optimum collagen pellet formation occurs. The

fluorescence values of the samples in relation to the following concentration, determined by fluoremetry, can also be found in Table 2.

Table 2: RIPA Buffer Concentration Series

RIPA Buffer (Original)	1.0% Nonidet-P40	0.2% SDS	Fluorescence Value (nm)
RIPA Buffer 1	8.0 % Nonidet-P40	0.9% SDS	34.231
RIPA Buffer 2	6.0 % Nonidet-P40	0.7% SDS	36.601
RIPA Buffer 3	4.0 % Nonidet-P40	0.5% SDS	32.734

RIPA Buffer 2, with 6.0% Nonidet-P40 and 0.7% SDS, showed the highest fluorescence value of pentosidine and these concentrations for the RIPA buffer were used for the remaining experiments.

FLUOROMETRY

The emission wavelengths for 21 skin samples were measured using fluorometry. The irises of the Perkin Elmer LS55 Fluorescence Spectrometer with FL WinLab Software were set at 2.5 entering and 2.5 exiting. The excitation wavelength was set at 355nm and emission wavelength was set at 422nm. The concentrations used for the pentosidine standard curve can be found in Table 3 and the resulting fluorescence values can be found in Table 4 below. From this table, a standard curve was graphed, as seen in Figure 4.

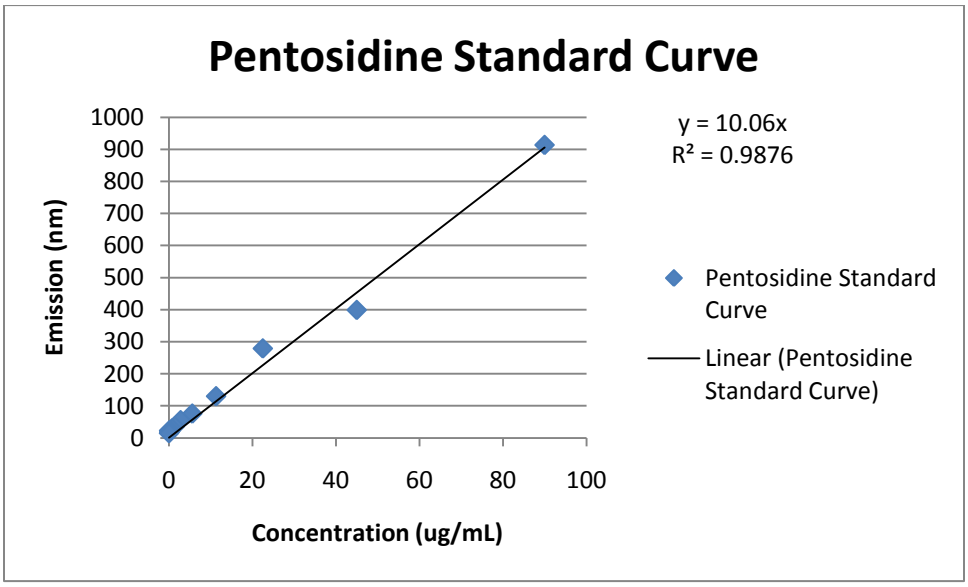
Table 3: Dilution and Concentration of Pentosidine Standard Curve

Dilution	Concentration
1:1	0.0900 mg/mL
1:2	0.0450 mg/mL
1:4	0.0225 mg/mL
1:8	0.0113 mg/mL
1:16	0.0056 mg/mL
1:32	0.0028 mg/mL
1:64	0.0014 mg/mL
1:128	7.0313×10^{-4} mg/mL

Table 4: Pentosidine Fluorescence Values for Pentosidine Standard Curve

Pentosidine Concentration (ug/mL)	Fluorescence Value (nm)
90	914.05
45	399.36
22.5	279.31
11.3	130.34
5.6	76.039
2.8	55.509
1.4	35.863
0.703	29.289
0.35	22.025
0.176	20.349
0.0879	18.087
0.0439	23.162
0.022	15.1

Figure 4: Pentosidine Standard Curve



The coefficient of determination, R^2, is 0.9876 for the linear regression equation determined for the standard curve. Therefore, the model is considered 98.8% accurate at predicting future data. This equation was used to determine the concentration of pentosidine in the skin samples.

Spectroscopy can be used in the quantitative analysis using the Beer-Lambert Law, where the absorbance of the sample is expressed as $A=\varepsilon lc$. In the equation, A is absorbance, ε is the molar absorbtivity in L/mol x cm, l is the distance the light travels through the solution in cm, and c is the concentration of the absorbing species in mol/L (Zumdahl & Zumdahl, 2006). In this case, the absorbing species is pentosidine. If both ε and l are known, determining A for the solution would allow for the calculation of concentration of the absorbing species in the solution. A plot of absorbance versus known concentrations would then produce a standard curve from which the concentrations of unknown samples could be determined.

However, there are some limitations to the methodology used. Both low and high emissions tend to deviate from Beer's Law. To account for this, it is necessary to zero the y-intercept, as seen in Figure 4 above, so that a solution with zero concentration will result in an emission reading of zero. This slightly alters the concentration readings produced at high and low emissions, but allows for further analysis. If the y-intercept had not been zeroed, some of the samples would be calculated to contain negative concentrations of pentosidine, which is not possible. The recorded fluorescence values can be seen below in Table 5.

Table 5: Fluorescence Values for Common Loon Samples

Tufts Code	Fluorescence Values
TV 07-445	21.409
TV 08-169	48.459
TV 08-227	30.339
TV 08-243	23.964
TV 08-255	16.527
TV 08-283	17.862
TV 09-031	17.769
TV 09-035	16.753
TV 09-036	7.065
TV 09-042	5.790
TV 09-062	2.989
TV 09-083	23.720
TV 09-092	4.453
TV 09-093	7.735
TV 09-095	8.880
TV 09-104	5.809
TV 09-105	4.652
TV 09-106	5.693
TV 09-107	13.542
TV 10-001	19.025

By evaluating the pentosidine fluorescence values using the linear regression equation from the standard curve evaluation, the concentration of pentosidine in the samples was determined using the following method:

Calculation using TV 07-445:
$y = 10.06x$
$(21.409) = 10.06\ x$
$x = 2.128$ ug/mL pentosidine

Therefore, the proposed pentosidine concentration for TV 07-445 is 2.128 ug/mL. Table 6 contains the determined concentrations of pentosidine for the samples.

Table 6: Experimental Concentrations of Pentosidine for Samples

Tufts Code	Pentosidine Concentration (ug/mL)	Estimated Age at time of Death
TV 07-445	2.128	
TV 08-169	4.817	2-3 weeks
TV 08-227	3.016	5-6 weeks
TV 08-243	2.382	
TV 08-255	1.643	Adult
TV 08-283	1.776	12-14 weeks
TV 09-031	1.766	Young
TV 09-035	1.665	2-3 years old
TV 09-036	0.702	
TV 09-042	0.576	Approximately 13 years old
TV 09-062	0.297	Adult
TV 09-083	2.358	Adult
TV 09-092	0.443	Adult
TV 09-093	0.769	Adult
TV 09-095	0.883	Adult
TV 09-104	0.577	Adult
TV 09-105	0.462	Adult
TV 09-106	0.566	Adult
TV 09-107	1.346	Adult
TV 10-001	1.891	Adult

COLLAGEN ASSAY

When the collagen assay was performed with the rat tail collagen, accurate results were not obtained. It appeared that the cross linked collagen threads adhered to the sides of the microfuge tube rather than solubilizing with the acetic acid and Direct Red 80 dye. As a result, the collagen was washed away along with the dye and we did not obtain a collagen reading using the spectrophotometer.

The procedure was attempted another time with the same results. After multiple trials, it was determined that the rat tail collagen would not solubilize in the glacial acetic acid. A different stock solution of acetic acid was used, but the collagen remained cross linked. This procedure was then attempted using collagen from bovine achilles tendon from Sigma, with the

same results. Because the bovine achilles tendon collagen could not be solubilized, a collagen standard curve could not be determined. As a last attempt, the assay was run using 0.1% collagen pre-solubilized in 0.1N acetic acid. The Type I collagen solution from calf skin was obtained from Sigma. When the Direct Red 80 dye was removed and the absorbance was read, an absorbance reading was not obtained for any of the concentrations.

Each time this procedure was conducted, concentrations for a standard curve were established using a series of two-fold dilutions, and the samples were run through the assay. After the samples were spun for 15 minutes in an Eppendorf 5415D microcentrifuge at 13,200 rpm, no visible collagen pellet had formed. Regardless, the supernatant was carefully removed and Direct Red 80 dye was added to the microcentrifuge tube. The tubes were then placed in a shaker, and then centrifuged to dye the collagen in the sample. Each time this step was conducted with collagen that was solubilized in acetic acid and the dye was removed, it appeared that the thread-like fibers either adhered to the sides of the tube. When this procedure was attempted with pre-solubilized collagen, there was no visual presence of any collagen at all. Therefore, when the alkali reagent was added and the absorbance read, the absorbance values for the samples were zero when compared to the distilled water blank.

It is unclear why the rat tail or bovine Achilles tendon collagen would not solubilize in the acetic acid, or why the pre-solubilized collagen did not interact with the dye. As directed by Biocolor Ltd. (2007), the collagen was solubilized in 0.5 M acetic acid to make a final concentration of 500 ug/mL. When first conducted, the white collagen threads were still visible congregated together within the acetic acid. The solution was pipeted up and down, heated using a hot plate at 100°C, and then left in a hot water bath at 65°C for two weeks. After this time, the collagen still hadn't solubilized, eliminating the possibility of creating an accurate standard

curve. When Biocolor Ltd. was contacted regarding the lack of collagen solubilization, technical support replied stating that the problem was due to the fact that authentic products were not being used. Technical support was unable to provide any other advice.

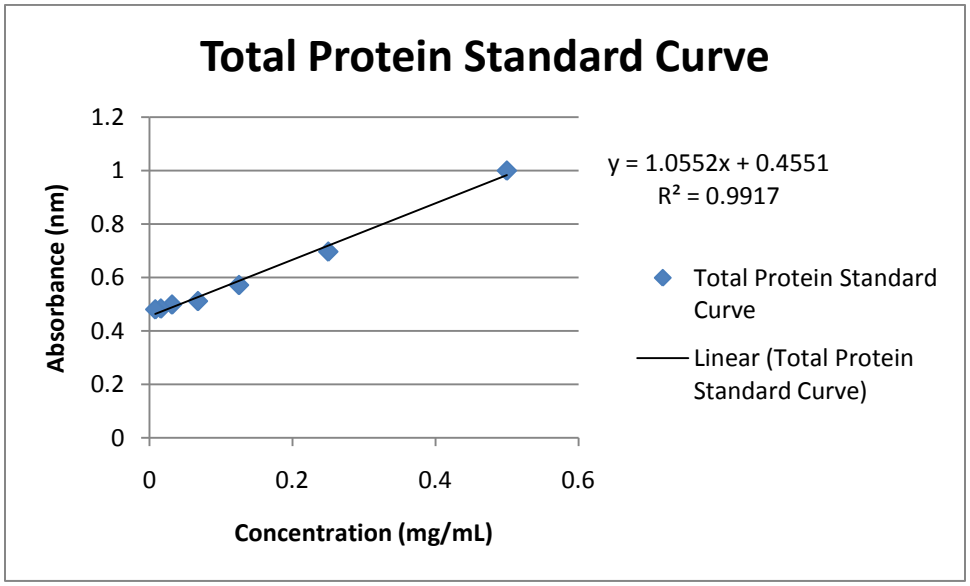
BRADFORD ASSAY

Table 6 illustrates the absorbance readings obtained for the serial dilution of 2 mg/mL Bovine Serum Albumin (cat # 23209) performed using the Bradford Assay. Figure 5 shows the standard curve obtained using these values.

Table 6: Absorbance Values for the Standard Curve Total Protein Concentrations

Concentration of BSA (mg/mL)	Absorbance (nm)
0.500	0.999
0.250	0.696
0.125	0.571
0.068	0.511
0.031	0.498
0.016	0.483
0.0078	0.480

Figure 5: Total Protein Concentration Standard Curve using the Bradford Assay



The coefficient of determination, R^2, is 0.9917 for the linear regression equation determined for the standard curve. Therefore, the model is considered 99.17% accurate when used to predict future data. This equation was used to determine the concentration of total protein in the skin samples. The Bradford Assay was used to determine the absorbance values for 18 samples, located in Table 7. All samples had to be diluted in order to fall within the linear range of the Bradford Assay (1nm to 0.100nm).

Table 7: Spectrophotometric Readings for Samples using Bradford Assay

Tufts Sample Code	Dilution	Absorbance
TV 07-445	1 : 4	0.726
TV 08-169	1 : 4	0.917
TV 08-227	1 : 4	0.650
TV 08-243	1 : 4	0.719
TV 08-255	1 : 4	0.609
TV 08-283	1 : 4	0.997
TV 09-031	1 : 4	0.845
TV 09-035	1 : 4	0.665
TV 09-036	1 : 4	0.712
TV 09-042	1 : 4	0.644
TV 09-062	1 : 4	0.926
TV 09-083	1 : 16	0.626
TV 09-092	1 : 4	0.666
TV 09-093	1 : 4	0.747
TV 09-095	1 : 4	0.718
TV 09-105	1 : 8	0.579
TV 09-106	1 : 4	0.821
TV 10-001	1 : 4	0.968

The total protein concentrations for the samples, as seen in Table 8, were estimated using the equation generated from the standard curve in Figure 4. These values were obtained using the following method:

For TV 07-0445,
$y= 1.0552x + 0.4551$
$0.726= 1.0552x + 0.4551$
$x= 0.2567$ mg/mL

Because the sample was diluted 1:4, the x value obtained is then multiplied by four. This gives the final estimated concentration of 1.0273 mg/mL.

Table 8: Estimated Concentrations of Total Protein in Samples

Tufts Sample Code	Estimated Concentration (mg/mL)
TV 07-445	1.0273
TV 08-169	1.7510
TV 08-227	0.7388
TV 08-243	1.0004
TV 08-255	0.5834
TV 08-283	2.0542
TV 09-031	1.4780
TV 09-035	0.7957
TV 09-036	0.9738
TV 09-042	0.7161
TV 09-062	1.7851
TV 09-083	2.5914
TV 09-092	0.7995
TV 09-093	1.1065
TV 09-095	0.9966
TV 09-105	1.9978
TV 09-106	1.3870
TV 10-001	1.9443

Using the Pentosidine concentrations located in Table 6 and the total protein concentrations in Table 8, the ratio of these two values can be obtained for each sample. This data can be found in Table 9.

Table 9: Ratio of Pentosidine Concentration to Total Protein Concentration

Tufts Code Sample	Ratio of Pentosidine (mg/mL) to Total Protein (mg/mL)	Estimated Age at Time of Death
TV 07-445	2.0714	
TV 08-169	2.7510	2-3 weeks
TV 08-227	4.0823	5-6 weeks
TV 08-243	2.3811	
TV 08-255	2.8162	Adult
TV 08-283	0.8646	12-14 weeks
TV 09-031	1.1949	Young
TV 09-035	2.0925	2-3 years old
TV 09-036	0.7209	
TV 09-042	0.8044	Approximately 13 years old
TV 09-062	0.1664	Adult
TV 09-083	0.9099	Adult
TV 09-092	0.5541	Adult
TV 09-093	0.6950	Adult
TV 09-095	0.8860	Adult
TV 09-105	0.2312	Adult
TV 09-106	0.4081	Adult
TV 10-001	0.9726	Adult

There does not appear to be any general correlation between the ratio determined and the estimated age of the bird (Appendix A) using the Bradford Assay. It was determined that this is most likely due to the evidence suggesting the Bradford Assay is insensitive to collagen (Dunhamel 1981).

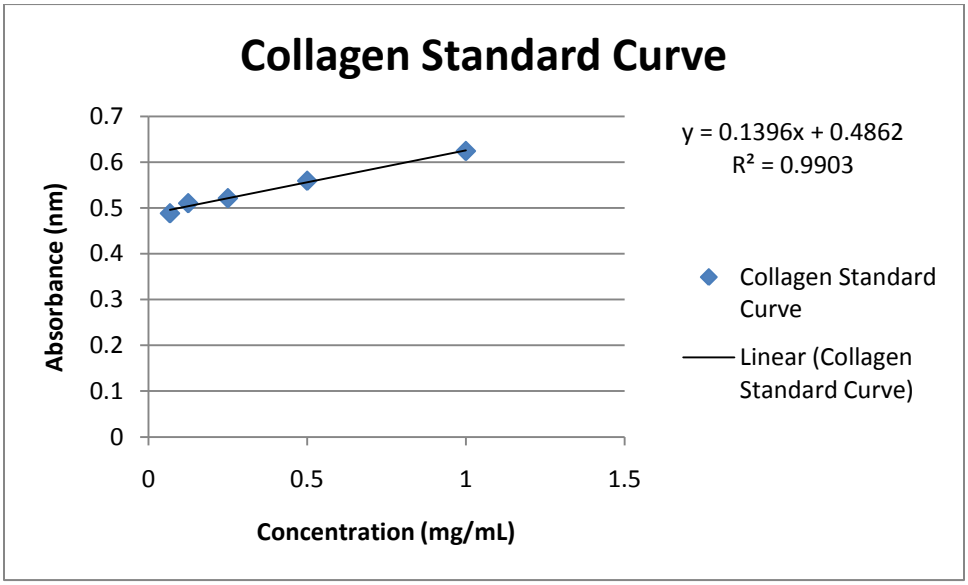
ALTERED BRADFORD ASSAY

Table 10 illustrates the absorbance readings obtained for the serial dilution of 1mg/mL type I collagen solution from calf skin performed using the altered Bradford Assay. Figure 6 shows the standard curve obtained using these values.

Table 10: Absorbance Values for the Standard Curve Collagen Concentrations

Concentration (mg/mL)	Absorbance (nm)
1.00	0.624
0.50	0.559
0.25	0.521
0.125	0.510
0.0675	0.488

Figure 6: Collagen Standard Curve Using Altered Bradford Assay



The coefficient of determination, R^2, is 0.9903 for the linear regression equation determined for the standard curve. Therefore, the model is considered 99.03% accurate when used to predict future data. This equation was used to determine the concentration of collagen in the skin samples. The altered Bradford Assay was used to determine the absorbance values of 18 samples, located in Table 11. Again, all samples had to be diluted in order for the absorbance readings to be measured within the linear range of the altered Bradford Assay (1nm to 0.100nm).

Table 11: Absorbance Values for the Skin Samples using Altered Bradford Assay

Tufts Sample Code	Dilution	Absorbance
TV 07-445	1 : 4	0.660
TV 08-169	1 : 4	0.669
TV 08-227	1 : 4	0.596
TV 08-243	1 : 4	0.609
TV 08-255	1 : 4	0.752
TV 08-283	1 : 4	0.784
TV 09-031	1 : 4	0.659
TV 09-035	1 : 4	0.613
TV 09-036	1 : 4	0.836
TV 09-042	1 : 4	0.750
TV 09-062	1 : 4	0.680
TV 09-083	1 : 8	0.648
TV 09-092	1 : 4	0.688
TV 09-093	1 : 4	0.620
TV 09-095	1 : 4	0.650
TV 09-105	1 : 4	0.789
TV 09-106	1 : 4	0.884
TV 10-001	1 : 4	0.997

Using the standard curve equation in Figure 6, Table 12 shows the estimated collagen concentrations for the samples. These values were determined using the following method:

For TV 07-0445,
$y = 0.1396x + 0.4862$
$0.660 = 0.1396x + 0.4862$
$x = 1.245$ mg/mL

Because the sample was diluted 1:4, the x value obtained is then multiplied by four. This gives the final estimated concentration of 4.980 mg/mL.

Table 12: Estimated Concentrations of Collagen in Samples using Bradford Assay

Tufts Sample Code	Estimated Concentration (mg/mL)
TV 07-445	4.980
TV 08-169	5.238
TV 08-227	3.146
TV 08-243	3.519
TV 08-255	7.616
TV 08-283	8.533
TV 09-031	4.951
TV 09-035	3.633
TV 09-036	10.02
TV 09-042	7.559
TV 09-062	5.553
TV 09-083	4.636
TV 09-092	5.782
TV 09-093	3.834
TV 09-095	4.693
TV 09-105	8.676
TV 09-106	11.40
TV 10-001	14.64

Using the Pentosidine concentrations located in Table 6 and the collagen concentrations in Table 12, the ratio of these two values were obtained for each sample. This data can be found in Table 13.

Table 13: Ratio of Pentosidine Concentration to Collagen Concentration using Altered Bradford Assay

Tufts Code Sample	Ratio of Pentosidine (mg/mL) to Collagen (mg/mL)	Estimated Age at Time of Death
TV 07-445	4.299	
TV 08-169	9.251	2-3 weeks
TV 08-227	9.644	5-6 weeks
TV 08-243	6.810	
TV 08-255	2.170	Adult
TV 08-283	2.093	12-14 weeks
TV 09-031	3.589	Young
TV 09-035	4.611	2-3 years old
TV 09-036	0.705	
TV 09-042	0.766	Approximately 13 years old
TV 09-062	0.538	Adult
TV 09-083	5.116	Adult
TV 09-092	0.770	Adult
TV 09-093	2.017	Adult
TV 09-095	1.892	Adult
TV 09-105	0.536	Adult
TV 09-106	0.499	Adult
TV 10-001	1.300	Adult

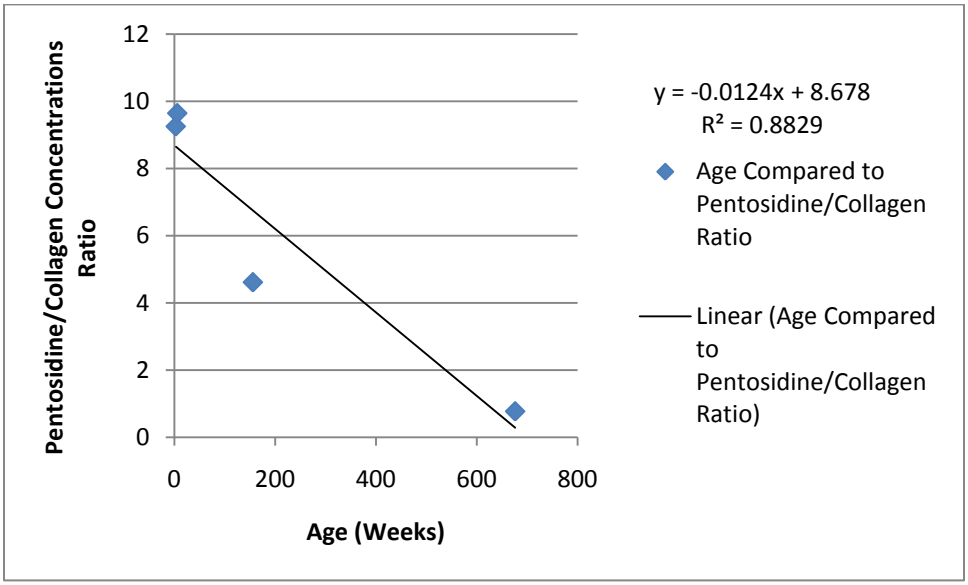
When comparing ratios in the above table to the estimated age of sample birds, it appears that the general trend is negatively correlated. This is not consistent with our hypothesis. It is possible that since total protein concentration was used to define the collagen concentration, there is a large amount of background protein that is being detected. The correlation between the pentosidine/collagen ratio and the age of the specimen can be quantified more accurately by evaluating the specimens of known age. Table 14 highlights this information.

Table 14: Specimens of Known Age and the Corresponding Pentosidine/Collagen Ratio

Tufts Code Sample	Ratio of Pentosidine Concentration (mg/mL) to Collagen Concentration (mg/mL)	Age (Weeks)
TV 08-169	9.251	3
TV 08-227	9.644	6
TV 09-035	4.611	156
TV 09-042	0.766	676

Using this data a standard curve was created to illustrate the correlation between the ratio of pentosidine to collagen concentration and the age of the specimens.

Figure 6: Standard Curve and Generated Equation



As can be seen in the figure above, the linear regression indicates a negative correlation between pentosidine/collagen ratio and the age of the specimens. The coefficient of determination, R^2, is 0.8829 for the linear regression equation determined for the standard curve. Therefore, the model is considered 88.29% accurate when used to predict future data.

CONCLUSION

It was originally hypothesized that pentosidine concentration would increase with increasing age in the Common Loon, and possibly other avian species. The main goal of this project was to develop a cost and time-effective assay using this correlation to determine the age of individual Common Loons. Although it appears as if the data collected do not support the hypothesis that pentosidine concentration increases with age in avian species, this is not necessarily true. In order to draw any conclusions between different samples, the ratio of pentosidine concentration to collagen concentration in the samples needs to be determined and then compared. This is due to the fact that collagen concentration varies dramatically over the lifespan of an organism (Chaney et al., 2001). In the theoretical final assay, the comparison of the pentosidine/collagen ratio would be used in all calculations and comparisons, not the interpolated pentosidine concentration. It is possible that a positive correlation could be detected using the final ratios. However, this could not be determined in this study.

All calculations were based on pentosidine and collagen standard curves. A few hindrances arose in the creation of these standard curves. In constructing the pentosidine standard curve, some of the data points fell below the linear range of the equation. Due to these low fluorescence values, as indicated by shading in Table 6, when using Beer's Law to calculate concentration from absorbance, it was necessary to interpolate some of the data and a y-intercept of zero was forced for the standard curve. This could have caused some of the discrepancies, such as the different fluorescence values obtained for delipidated and non-delipidated samples from the same specimen, as seen in Table 1. Although there are observed difference between the delipidated and non-delipidated samples, the difference is not significant due to the scale of the fluorence values used for this study. If the interpolation was not necessary and the standard

curve covered the entire range of the data points, then the differences could potentially be minimized further.

Many of the issues that arose during this study were due to difficulty in creating a true collagen standard curve. The pre-solubilized collagen purchased was prepared with acetic acid as instructed. The varying concentrations were then mixed with the Direct Red 80 dye. A dye-collagen complex did not form, therefore accounting for the lack of pellet formation. The Direct Red 80 dye, also known as Sirius Red, was purchased by Sigma rather than through Biocolor Ltd in the assay kit. However, since it is the same dye, the interaction should theoretically be the same. According to Kano et al. (2001), Sirius Red is one of the best understood techniques of collagen histochemistry and is widely used. At this point, it is unknown why the dye would not react with the different sources of collagen. The dye binds to the collagen fibers through an anionic interaction and is then read at the maximum absorbency, which is known to be at 540 nm. It appears that this anionic interaction did not occur, but it is not known why.

In accordance with this limitation, and the inconclusive pentosidine concentration data, several recommendations for future study have been proposed. First, it is imperative to determine a way in which to create an accurate collagen standard curve and use this to determine pentosidine/collagen ratios. With this, individual samples could be compared more accurately. Several possibilities when considering this recommendation include altering the dye used in the collagen assay, the type of collagen used for the standard curve, or exploring other possible methods.

Additionally, a separate experiment should be performed in order to determine pentosidine/collagen decomposition rates in avian species, or other factors that might alter concentrations. Although Fallon *et al.* (2006) suggest that pentosidine degradation is not an

issue, the results may not be extrapolated to other species or methods of storage. The samples collected in this study vary significantly in both condition and time between death and freezing. All samples were collected post-mortem and brought to Tufts upon discovery. The time between death and discovery varies significantly among samples, therefore leading to varying conditions of the specimens. In some samples, there was evidence of scavengers eating away at the carcass before collection or post-mortem fungal infections. Maggots were found within the body cavity of several samples. Other samples, on the other hand, appeared untouched post-mortem upon necropsy. These varying conditions are common amongst wildlife specimens, which are exposed to the elements post-mortem. It is not possible to determine the exact conditions that the specimen was exposed to before discovery, making it difficult to account for the current condition or to try to standardize specimen condition.

After initial discovery of the specimen, there is no established protocol dictating storage conditions while in transport to the Wildlife Clinic. Most samples are frozen before arrival at the clinic, stored in a refrigerator and thawed for necropsy, then either stored in the refrigerator or a standard freezer. Samples were saved for this study in both the refrigerator and freezer, depending on time between necropsy and collection. Once brought back to WPI, the samples were labeled and placed in a -20°C freezer until needed. The varying storage conditions could affect decomposition rate. If decomposition breaks down pentosidine, or the half life of pentosidine is less than previously determined, this could severely alter calculated concentrations. This factor would require steps to compensate for this decrease in concentration. If the time between death and retrieval of samples could be recorded, it is possible to determine a way to account for this when making calculations.

Pentosidine accumulation is affected by both diet and upbringing in either captivity or in the wild. Restrictive diets, such as the need to forage, are common among loons and other wild birds. According to Fallon (2003), this diet would decrease AGE accumulation. However, the exercise required when foraging leads to oxidative damage, which would then increase AGE accumulation. If these factors differed on an individual basis, varying with environmental conditions, pentosidine concentration would then be affected both by age and living conditions. This additional factor could account for differences seen in the data. However, it would be a challenge to try to control for this factor in an assay. It is expected that due to similar environment, diet, and lifespan, pentosidine accumulation would not differ significantly within a species. To test for this, banded samples from different locations could be collected and pentosidine concentrations could be compared. If there is a difference among locations, this would need to be accounted for in the assay.

In the future, a study could be conducted to assess any discrepancies in data due to the use of deceased specimens versus live specimens. If an assay were run on samples from both live and deceased birds, the two groups could be compared. If there is a discrepancy between the values calculated for live birds and deceased birds, then further investigation into the role of specimen condition is needed. The original goal of this study was to create an assay to age live birds. Deceased birds were used for ethical reasons, since two grams of skin were needed in the assay. The ability to age deceased birds would help in research, but would not have as much of an impact in the clinical environment. In this respect, it is more important to develop the assay for live birds. If the amount of skin needed could be decreased to that required for a punch biopsy, this assay could be tested on live birds.

If the original goal of this project could be attained, this assay could have vast applications for wildlife conservation and medicine, ornithologists, and researchers in general. In particular, the Tufts Wildlife Clinic could use this assay to determine age of patients, which would provide critical information in terms of care and possible recovery. Studies could also be conducted to determine any trends in age of loons and deaths caused by lead poisoning, fungal infections, or other common causes of death. This assay could also lay the foundation for other possible assays which can be used to age other avian species.

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APPENDIX A: INDIVIDUAL BIRD DATA

Tufts Code	Death	Sex	Proposed Cause of Death
TV 07-445	2007		
TV 08-169	2008	Young 2-3 week old Female	Skinny; died at the wild life clinic; Severe Fungal Infection
TV 08-227	2008	Young 5 – 6 week old Male	GI Infection
TV 08-243	2008		BANDED
TV 08-255	2008	Adult Male	Healed Fractured Radius from gunshot; fractured humerus; Euthanized
TV 08-283	2008	Young 12 – 14 week old Male	Blunt Trauma (possible boat hit)
TV 09-031	2009	Young Male	Possible drowning
TV 09-035	2009	2 – 3 year old Female	Beached
TV 09-036	2009		
TV 09-042	2009	~13 year old Female	MAYBE BANDED
TV 09-062	2009	Adult Female	Drowned in net
TV 09-083	2009		
TV 09-092	2009	Adult Female	Severe parasitic infection
TV 09-093	2009	Male	Lead poisoning
TV 09-095	2009	Adult Male	swallowed spoon; cut through gizzard and caused infection and death
TV 09-104	2009	Adult Female	Lead poisoning
TV 09-105	2009	Adult Male	Shot in head
TV 09-106	2009	Adult Male	Possible fight with another loon causing trauma to abdomen which hit bowel and caused infections
TV 09-107	2009	Adult Male	Possible blunt trauma
TV 10-001	2010	Adult Male	Fungal infections; choking to death on crab when brought into clinic