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Foot web pentosidine does not covary strongly with age in four species of wild seabirds

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ABSTRACT

Age is an important parameter for a variety of ecological applications, including population viability analyses, contaminants monitoring and targeting of individuals for conservation. While many organisms can be aged by annual rings, dentition and other techniques (i.e., fish otoliths, clam growth rings, mammal tooth wear), there are no minimally invasive biomarkers for accurately aging birds in the wild. For the past century, banding has been the only way to identify a bird of known age, which requires continuous effort on a large scale with possibly low return rates. Recent studies have identified pentosidine as a potential biomarker of chronological aging in several bird species. To test this idea in four species of long-lived seabirds, we collected skin biopsies from the foot webs of previously banded, known-age seabirds: black-legged kittiwakes (*Rissa tridactyla*; 0–19 y old), Atlantic puffins (*Fratercula arctica*; 5–26 y old), razorbills (*Alca torda*; 0–15 d old) and thick-billed murres (*Uria lomvia*; 0–35 y old). Foot web samples were specifically chosen because this was the least invasive site for substantial skin biopsy. Samples were analysed with high performance liquid chromatography to quantify pentosidine levels. Collagen levels were estimated through hydroxyproline assays to normalize pentosidine content across individuals. Kittiwakes displayed a weak correlation ($r^2 = 0.20$) between age and pentosidine/collagen. Puffins (adults only, $r^2 = 0.02$), razorbills (chicks only, $r^2 = 0.08$), and murres (adults, $r^2 = 0.04$) did not show any associations with age. We concluded that pentosidine content in the foot web does not appear to be a reliable method for aging seabirds in the wild. An absence of change in pentosidine in the foot web with age is further evidence that long-lived seabirds may maintain physiological performance into old age.

1. Introduction

Age is one of the most important parameters determining the health, reproductive potential and survival of organisms (Nussey et al., 2008; Ricklefs, 2008; Jones et al., 2014). Thus, knowledge of the age of an animal is critical for conservation applications, including population viability analyses, contaminants monitoring, and targeting of individuals for intervention (Fallon et al., 2006; Cooey, 2008; Holmes and Martin, 2009). While many organisms can be aged by annual rings, dentition and other techniques (i.e., fish otoliths, clam growth rings, mammal tooth wear), there are no biomarkers for accurately aging flying vertebrates in the wild beyond the first few years of life (Holmes and Martin, 2009; Herman et al., 2009; Munshi-South and Wilkinson, 2010; Jarman et al., 2015). Therefore marking a bird or bat during its hatch or birth year has been the only way to identify its exact age (Lincoln, 1921; Holmes and Martin, 2009; Munshi-South and Wilkinson, 2010). While osteologically-based age determination techniques are well-known for other vertebrates (Bagenal, 1974; Peabody, 1961; Reid, 1981), including many mammals (e.g., Morris, 1972; Grue and Jensen, 1979), none is well established for birds or bats (Broughton et al., 2002; Munshi-South and Wilkinson, 2010). Although the number of endosteal lamellae in transverse thin sections of the tibiotarsus showed some promise with small sample sizes in birds (Klomp and Furness, 1992), the technique was inaccurate with a larger number of individuals from one of the same species (Broughton et al., 2002). Moreover, such a procedure requires euthanizing the bird. It is thus

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normally possible only to determine crudely whether birds are relatively young based on moult patterns, cranial pneumatization or morphometrics (e.g., McNeil and Burton, 1972; Sugimori et al., 1985; Siegel-Causey, 1989).

One reason why long-lived birds may be difficult to age in the wild is because they typically do not show the classic signs of physiological aging present in short-lived mammals. Actuarial senescence, the increasing rate of mortality with age, is more important in long-lived than short-lived wild animals as a higher proportion of mortality is attributable to senescence in the former relative to the latter (Ricklefs, 2008, 2010; Turbill and Ruf, 2010). Whereas relatively few individuals in short-lived species will live long enough to be senescent in the wild, a larger proportion of individuals in long-lived species will be senescent. Perhaps because of stronger selection for postponed physiological senescence in long-lived birds, studies of long-lived wild birds have detected few signs of declining immunity (Apanius and Nisbet, 2006; Lecomte et al., 2010), oxidative stress (Jimenez et al., 2019; Lecomte et al., 2010) or reproduction (Nisbet et al., 2002; Coulson and Fairweather, 2003; Lecomte et al., 2010) with age that routinely accompany physiological aging in mammals and short-lived birds (Cichon et al., 2003; Holmes and Ottinger, 2003; Saino et al., 2003; Moe et al., 2009; Palacios et al., 2007; these ideas are reviewed by Nisbet, 2001; Ricklefs, 2008, 2010; Holmes and Martin, 2009).

Aging is a complex phenomenon in which, at the cellular level, harmful changes can accumulate with time in each organism until its inevitable death. Biochemical changes at the level of cells and organelles ultimately result in organismal death (Harman, 1992, 2009; Martin et al., 1996). For example, the free radical theory of aging proposes that the generation of free radicals within the mitochondrial electron transport chain leads to progressive damage to organs, biological aging and, eventually, death (Harman, 1992; Wickens, 2001; Monaghan et al., 2009). Indeed, oxidative stress is speculated as a contributing factor in a number of aging-related diseases (Valko et al., 2007; Lushchak, 2014; Daffu et al., 2013). Another possible explanation of aging is the cross-linking theory of aging, where sugars modify body proteins through the Maillard reaction (Monnier and Cerami, 1981; Grandhee and Monnier, 1991; Iqbal et al., 1999a,b). The results of cross-linking are advanced glycation end products (AGEs) whose formation is accelerated by oxidative stress (Wickens, 2001). Advanced glycation endproducts can damage the organism directly, by modifying intracellular or extracellular proteins, and, indirectly, by binding to the receptor of advanced glycation end products (RAGE). Ligand binding to RAGE increases intracellular reactive oxygen species formation, and therefore leads to oxidative stress and could contribute to aging as described by the free radical theory (Daffu et al., 2013; Nowotny et al., 2015). One such AGE is pentosidine (Sell and Monier, 1989) and named after originally being synthesized from pentose—although pentosidine can be derived from other sugars. At the end of the Maillard reaction, skin pentosidine cross-links the arginine and lysine residues within collagen (Sell and Monnier, 1989; Sell et al., 1998; Dyer et al., 1991). Cross-linking alters collagen's structure and mechanical properties, which makes the protein less degradable by proteases (Nowotny et al., 2015). Furthermore, human neurons exposed to pentosidine increased their reactive oxygen species generation and apoptotic cell death (Nitti et al., 2005). Thus, AGEs could link biological age (physiological state) to chronological age (time since birth) (Sell et al., 2003; Chaney et al., 2003; Fallon et al., 2006). Indeed, due to its stable, fluorescent, and accumulating properties, previous studies confirmed pentosidine as potential biomarker of age in mammals, birds, and, most recently, in turtles (Sell et al., 1998; Cooey et al., 2010; Dammann et al., 2011; Iverson et al., 2017).

Pentosidine increases significantly with age in chickens (Iqbal et al. 1997, 1999a), California gulls (*Larus californicus*; Chaney et al., 2003), ruffed grouse (*Bonasa umbellus*; Fallon et al., 2006), monk parakeets (*Myiopsitta monachus*; Cooey et al., 2010) and double-crested cormorants (*Phalacrocorax auritus*; Fallon et al., 2006; Cooey, 2008; Dorr et al., 2017), but not common gulls (*Larus canus*; Rattiste et al., 2015), kakapos (*Strigops habroptilus*; Campion, 2015) or bridled terns (*Onychoprion anaethetus*; Labbé et al., 2019). Thus, results so far are consistent with the idea that physiological senescence (as shown by pentosidine content increasing with age) is evident in short-lived but not long-lived birds, but few long-lived birds have been tested. In cormorants, pentosidine levels in the patagium were lower than breast skin, but both tissues showed a similar increase with age (Cooey et al., 2010). Levels in foot webs were also lower than in breast skin (Chaney et al., 2003). We examined levels in the foot web, to determine if biopsies from the foot web could be a non-lethal and minimally invasive technique for measuring age in waterbirds, as biopsies from the foot web may cause less damage and risk of infection than biopsies from the breast or patagium. We examined whether pentosidine can be used as a biomarker of chronological age in four species of long lived seabirds: black-legged kittiwakes (*Rissa tridactyla*), Atlantic puffins (*Fratercula arctica*), razorbills (*Alca torda*), and thick-billed murres (*Uria lomvia*). We expected pentosidine concentrations to increase with age among all individuals, with species-specific rates varying with maximum lifespan—as found in other taxa (Fallon et al., 2006).

2. Materials and methods

2.1. Tissue sampling

Four species of seabirds were sampled during their breeding season in 2015 and 2016 under McGill animal use protocol 2015-7599. Only birds banded as chicks, and thus of known ages, were targeted. Adults were trapped by various methods and put in cloth bags to minimize stress. Middleton Island, Alaska (59° 26′ N, 146° 20′ W), in the Gulf of Alaska (Gill and Hatch, 2002), is the site of an abandoned U.S. Air force defense radar tower that black-legged kittiwakes use for nesting. The buildings' sides were upgraded with wooden panels, nest ledges, and sliding panes of one-way mirror glass, thus creating a research habitat consistently of hundreds of nest sites easy to access and monitor. Since 1984, kittiwake adults and chicks have been banded on Middleton Island during every breeding season (Gill and Hatch, 2002). At Middleton, kittiwake reproductive success increases until about age 12, and then decreases rapidly (Elliott et., 2014, with little change in muscle physiology Brown et al., 2019). In 2015, we collected samples from adults either nesting on or prospecting for sites on the tower, as well as from chicks hatched on it. Adults were captured opportunistically with wire hooks passed through slots in the wall at or below the level of their nests (Chivers et al., 2015). Chicks were monitored daily until 40 days since hatching, and were taken from their nest on the day of sampling.

Machias Seal Island, New Brunswick (44° 30′ N, 67° 06′ W) lies 18 km off the coast of Maine (Diamond and Devlin, 2003; Kelly et al., 2015). Since 1980 and 1995 respectively, Atlantic puffins and razorbills have been banded on Machias Seal Island (Kelly et al., 2015). In 2016, we collected samples from Atlantic puffins (adults and chicks) and razorbill chicks spread across the island. Puffins nest in burrows, and most adults were captured by extracting them directly from their nest sites. Occasional individuals were caught with drop-box traps or mist nets. While still in the egg stage, most study burrows were flagged and regularly checked until their hatch date. Each had an assigned sampling day and was not inspected until a few days before that date to minimize stress and abandonment by the parents (Rodway et al., 1996). Chicks younger than 36 days were caught in the burrow. Puffin chicks, older than 36 days, were caught at night during their first trip to the sea. Razorbills nest among boulders and adults are hard to catch, so we did not capture any adults. Razorbill chicks were monitored and sampled similarly to the puffin chicks at about age 10 d.

Coats Island, Nunavut (62° 57′ N, 82° 45′ W) is an island in northern Hudson Bay (Gaston et al., 1994). Thick-billed murres on Coats Island are located in two sub-colonies 1.5 km apart and have been banded since 1981 (Gaston et al., 1994; Elliott et al., 2015). At Coats, reproductive success increases until age 25, and then decreases slightly (Elliott et al., 2014). The oldest record for a thick-billed murre is 37

years (Jimenez et al., 2019), and our age range of 0-35 y is therefore representative of the age range present in the wild population. In 2016, adult murres were captured using a noose pole during the incubation period. Murre chicks were also caught by noosing near the top of the cliffs within 5 days of their hatch.

For all species, feet were cleaned with isopropanol, followed by puncturing an interdigital foot web with a 6 mm biopsy punch. The collected skin sample was stored in a labeled centrifuge tube. We collected one sample per bird, punching through the entire web. The foot hole was treated with antibiotic ointment to prevent infection and pressed with styptic powder in between two cotton balls until hemostasis. The full procedure lasted for a maximum of 10 min before releasing the bird. Samples were transported, surrounded by icepacks, from the field to a −20 °C freezer within 5 to 60 min after being collected. From 3 weeks to 3 months later, they were transported with dry ice to our laboratory facilities. Sex was already known for black-legged kittiwakes based on copulation position and for thick-billed murres based on genetics.

2.2. Sample processing

Regardless of species, all samples were processed identically in a method modified from that described by Iqbal et al. (1997), Chaney et al. (2003), and Fallon et al. (2006). Biopsies were scraped clean, minced with razor blades and transferred to glass tubes. To remove lipids from skin biopsies, each tube was filled with 2 mL of a 2:1 chloroform/methanol solution, placed in a shaker, and left mixing at 4–6 °C for 18 h. We discarded the chloroform/methanol waste by pipetting it out of the tubes and leaving the rest to air-dry for 1 h. Once completely dry, the residue was rehydrated, hydrolyzed in 1 mL of 6 M HCl and heated to 110 °C for 18 h. After cooling down, the samples were centrifuged to settle the suspended remains and retain only the liquid portion. The skin extracts were then neutralized by titration with NaOH. The resulting saline solutions were frozen at −80 °C and transferred to a freeze dryer (Labconco 75040) to undergo sublimation. Finally the samples were reconstituted in 1 mL of Milli-Q water and filtrated with 0.22 μm syringe filters. We examined the effect of reconstituting the sample in 0.25–1.5 mL of Milli-Q water, and there was no difference in the recovered pentosidine or collagen values among the different dilutions. Some spiked samples with pentosidine (from individuals not otherwise used in this study) were used to help identify peaks on HPLC chromatographs. The percent recovery of 0.080 pmoL/μL spiked pentosidine in five murre foot web samples was 99.7 ± 4.0 (SD) %.

2.3. Pentosidine measurement

Pentosidine (Cayman Chemicals, Ann Arbor, MI), in a crystalline solid state is stable for up to 2 years when kept at −20 °C (we obtained standard curves varying by < 5% when comparing pentosidine kept frozen over that time period). Once diluted in water, the aqueous solution should not be stored for more than a day (standard curves of pentosidine decreased by about 0.8% per hour such that apparent levels were 20% of actual levels after 100 h). Therefore, the stock was divided in multiple aliquots and frozen until needed during the experiment. Before each experiment, one aliquot was thawed and further diluted into 6 standard concentrations of 0.5 nmoL/L, 1.0 nmoL/L, 2.0 nmoL/L, 4.0 nmoL/L, 8.0 nmoL/L, and 16 nmoL/L. Similarly to the sample extracts, each standard was filtered with 0.22 μm syringe filters before its transfer to a glass vial.

Pentosidine was measured through modifications of the reverse phase high-performance liquid chromatography (HPLC) techniques previously described by Iqbal et al. (1997), Chaney et al. (2003), and Fallon et al. (2006). Pentosidine concentrations were estimated in duplicate using a Dionex UltiMate 3000 HPLC work station with an in-line fluorescence detector (UltiMate 3000, excitation 325 nm, emission 370 nm). The mobile phase consisted of acetonitrile, Milli-Q water and 0.01 M heptafluorobutyric acid (HFBA). Injections (100 μL) were separated using a linear gradient of 5–30% acetonitrile, in water with HFBA from 0 to 35 min using a C8 column (Acclaim 4.6 × 10 mm) and flowrate of 1 mL/min. The resulting elutions were visualized as chromatograms with peaks of different magnitudes at specific timespans. Integration of those peaks was performed in Chromeleon 7 version 7.1.2.1478 software package. A pentosidine standard was processed after every 4 to 5 skin extracts to confirm pentosidine's exact elution timing. The column was cleaned and equilibrated for a minimum of 20 and 15 min respectively between injections.

2.4. Collagen measurement

Assuming that 14% of collagen by weight is hydroxyproline, we used a spectrophotometric assay to measure each sample's hydroxyproline content using a commercial kit (MAK008, Sigma-Aldrich, Missouri, St Louis; Maekawa et al., 1970). Hydroxyproline was measured for all hydrolyzed skin extracts in duplicate (coefficient of variation averaged 8.9%) with a Fluostar omega microplate reader (emission wavelength of 560 nm). The results were examined with MARS Data Analysis version 2.41 software. The assay standards were diluted in water with 6 M NaCl to mimic the NaCl formed during sublimation of neutralized samples of hydrolyzed skin tissue; an initial attempt using KCl led to inaccurate data compared with water.

2.5. Statistical analysis

Statistical regression analyses using general linear models (LM) were carried out with R to determine the relationship between pentosidine or hydroxyproline concentrations and age + age^2 + sex. We considered an age^2 term to account for non-linear relationships. We included sex as a covariate only for murres and kittiwakes as they were the only two species where we had sex information. We used a backwards selection procedure where we included age, age^2 and sex as covariates and then dropped non-significant terms systematically based on their non-significance. We used a significance level of $P < 0.05$, and considered significant relationships with an $R^2 > 0.5$ as useful for age determination.

3. Results

3.1. Kittiwakes

We sampled 30 black-legged kittiwake adults aged 3–19 years, with most single age groups having two individuals per age. After normalizing across individuals using the hydroxyproline assay results, pentosidine concentrations in collagen ranged from 1.31 pmol pentosidine/mg collagen to 8.16 pmol/mg (Fig. 1). These concentrations increased linearly with age: pentosidine = 0.1689 ∗ age + 2.5015, $R^2 = 0.20$ ($t_{29} = 2.64$, $P = 0.01$; omnibus GLM age^2: $t_{27} = 1.49$, $P = 0.15$; sex: $t_{27} = 0.42$, $P = 0.69$). Average pentosidine concentrations in each group increased linearly with age: pentosidine = 0.1579 ∗ age + 2.5797, $R^2 = 0.39$ ($t_{15} = 3.02$, $P = 0.009$) (Fig. 1). We sampled 4 black-legged kittiwake chicks aged of 10, 14, 40, and 44 days. Their pentosidine concentrations in collagen ranged from 1.84 pmol/mg to 10.45 pmol/mg (Fig. 1). Like in the adult kittiwakes, the pentosidine levels increased linearly with age: pentosidine = 0.1642 ∗ age + 0.2621, $R^2 = 0.54$ ($t_3 = 1.52$, $P = 0.27$). When we pooled adults and chicks, pentosidine levels were independent of age (age: $t_{31} = -0.05$, $P = 0.96$; age^2: $t_{31} = 0.49$, $P = 0.63$; sex: $t_{31} = 0.44$, $P = 0.66$; $R^2 = 0.06$). However, if we removed one chick, the relationship improved considerably (age: $t_{30} = 3.07$, $P = 0.004$; age^2: $t_{30} = -0.82$, $P = 0.42$; sex: $t_{30} = 0.72$, $P = 0.74$; $R^2 = 0.23$). Collagen content from the standardized 6 mm skin biopsy increased with age in chicks (albeit not significantly) but not in adults (chicks: age: $t_3 = 3.01$, $P = 0.09$; age^2: $t_2 = 0.14$, $P = 0.91$; adults: age: $t_{27} = 0.68$, $P = 0.50$; age^2: $t_{27} = -0.48$, $P = 0.63$; sex: $t_{27} = 0.54$, $P = 0.60$; Fig. 2).

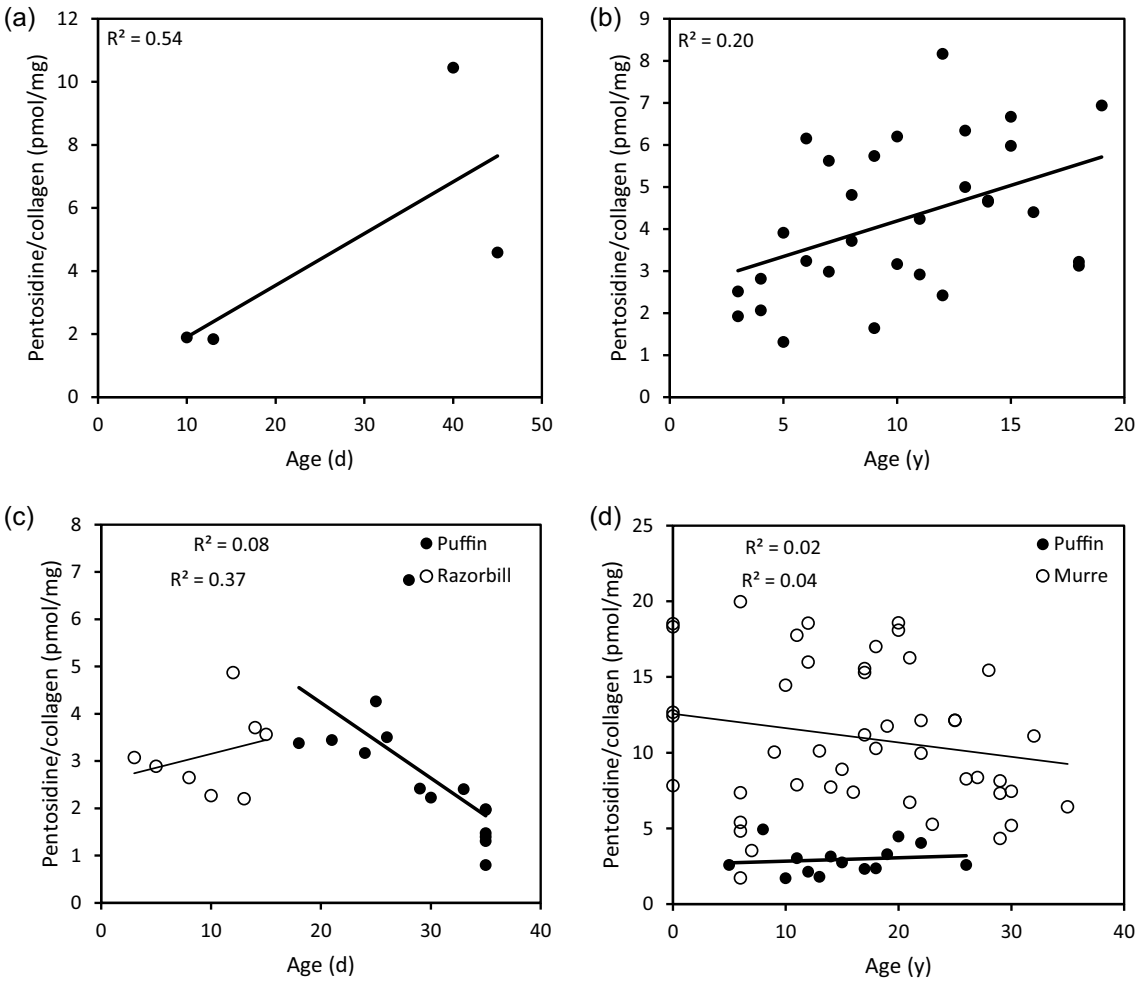

Fig. 1. Pentosidine/collagen (pmol/mg) relative to age in (a) black-legged kittiwake chicks, (b) black-legged kittiwake adults, (c) auk chicks (puffins and razorbills) and (d) auk adults (puffins and murres; includes murre chicks of unknown exact age).

3.2. Auks

We sampled 14 adult puffins aged 5–26 years and 15 chicks aged 15 to > 35 days old. All age groups were represented by one adult, but no chicks aged 19, 20, 22, 23, 27, 31, 32, and 34 days were captured. Pentosidine concentrations did not vary with age in adults (R^2 = 0.02, age: t_{12} = 0.47, P = 0.65; age^2: t_{12} = 0.44, P = 0.67; Fig. 1). Concentrations decreased linearly with age in the chicks: pentosidine = −0.1545 ∗ age + 7.3278 (R^2 = 0.39, age: t_{14} = −2.91, P = 0.01; age^2: t_{12} = −2.17, P = 0.05). There was no relationship for pooled chick and adult data (R^2 = 0.17, age: t_{42} = 1.43, P = 0.16; age^2: t_{42} = −0.64, P = 0.52). Collagen content of 6 mm biopsies increased with age in chicks but not adults (chicks: age: t_{13} = 3.81, P = 0.002; age^2: t_{12} = 1.08, P = 0.30; adults: age: t_{11} = 0.8, P = 0.42; age^2: t_{11} = −0.80, P = 0.44; Fig. 2). We sampled 8 razorbill chicks aged 3–15 days and pentosidine concentrations did not vary with age (R^2 = 0.08; age: t_7 = 0.74, P = 0.49; age^2: t_7 = 0.55, P = 0.61). Collagen increased with age, albeit not significantly (age: t_6 = 1.61, P = 0.16; age^2: t_5 = 0.65, P = 0.54; Fig. 2). We sampled 5 thick-billed murre chicks of unknown ages (< 8 d old) and 37 adults between 6 and 32 years of age. For pooled data (chicks and adults), pentosidine concentrations did not vary with age (R^2 = 0.04, age: t_{43} = −1.30, P = 0.20; age^2: t_{43} = −1.13, P = 0.27; sex: t_{43} = 0.70, P = 0.49) (Fig. 1). No trend was apparent for chicks and adults separately (Fig. 1). Collagen was higher in adults than chicks (age: t_{44} = 2.87, P = 0.006; age^2: t_{43} = −1.44, P = 0.16; Fig. 2).

4. Discussion

Unlike some previous studies on primarily short-lived species (Iqbal et al. 1999a; Chaney et al., 2003; Fallon et al., 2006; Cooey et al., 2010; Dorr et al., 2017), pentosidine concentrations in collagen did not increase substantially with age in the four long-lived seabird species examined in the current study. These results are consistent with the idea that long-lived birds do not show many of the classic traits of physiological aging present in mammals and short-lived birds (Nisbet, 2001; Ricklefs, 2008). Only kittiwakes (the shortest-lived species) showed a significant increase in pentosidine with age in adults, but there was no significant relationship for pooled chick and adults values. Common gulls and bridled terns, which are relatively long-lived, also showed no significant increase with age in pentosidine measured in biopsies from the wing patagium (Rattiste et al., 2015; Labbé et al., 2019), although California gull foot webs showed an increase (Chaney et al., 2003). The California gull study demonstrated lower pentosidine levels in the foot webs of young (~4 years) than old (~20 years) gulls, but showed no change with age within either age class. Without information from intervening age classes, such trends may represent environmental variation associated with each cohort. Skin collagen half-life is about 15 years (Iverson et al., 2017), and consequently the pentosidine technique may be less reliable in long-lived species, as implied by recent work on turtles (Iverson et al., 2017). Moreover, the foot web is more exposed to ultraviolet radiation than the breast, which could lead to degradation of pentosidine, and so levels are likely lower than other skin sites (as is the case for the patagium, Cooey et al., 2010) and trends

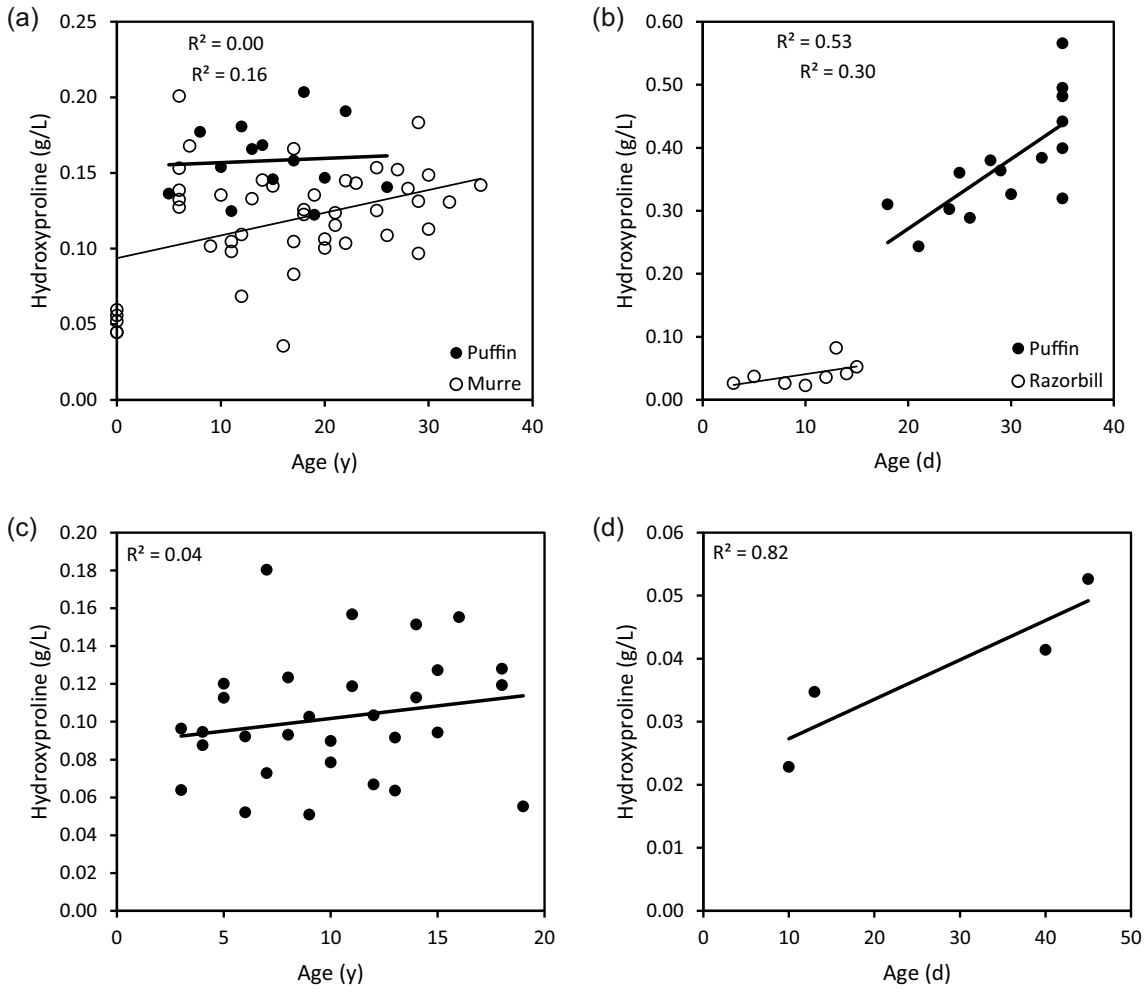

Fig. 2. Collagen content (g collagen/L of buffer) relative to age in (a) black-legged kittiwake chicks, (b) black-legged kittiwake adults, (c) auk chicks (puffins and razorbills) and (d) auk adults (puffins and murres; includes murre chicks of unknown exact age). Values are reported as hydroxyproline content from a 6 mm punch biopsy from the foot webbing of each individual (without accounting for tissue mass), which was converted to collagen by dividing by 0.14.

with age may be weaker. Collagen per sample increased with age, likely because the foot webs became thicker with age, and so more tissue was collected per sample of constant diameter.

We argue that pentosidine is unlikely to be a useful biomarker for chronological age in the foot web of birds. Iverson et al. (2017) found that "concentrations were too variable to permit precise estimates of age for turtles of unknown age" and the same appears to be true for volant vertebrates. The earlier successful papers (on breast or patagial skin tissue) primarily demonstrated an increase in pentosidine with age during the first few years of life. Indeed, our own data showed a relationship between pentosidine and age during chick development, which would be unexpected for a metric that is purported to provide age over an entire animal's lifespan. Chaney et al. (2003) showed higher pentosidine in California gulls aged 200–300 months than those aged about 50 months. Given that reproductive maturity is typically 48 months, this comparison was between gulls breeding for the first time and those that had bred for many years. Similarly, the increase in monk parakeets was primarily between those aged 6 months or less and those aged 20 months or more (Cooey et al., 2010) and the increase in double-crested cormorants was primarily between those younger than 50 months and those older than 50 months (Fallon et al., 2006; Cooey, 2008). Given that moult patterns can distinguish birds in their first year or two of reproduction, there seems little need for pentosidine to make such distinctions. Moreover, within a single family (gulls, Laridae), the patterns included no variation with age (Rattiste et al., 2015), an increase between young breeders and old breeders (Chaney et al., 2003) and no change between chicks and adults, but a weak increase with age in breeders (our study). Taken together, our results combined with the literature suggests that this technique does not appear to be reliable for precise aging of wild seabirds. This is likely especially true for foot web biopsies because foot web biopsies have high levels of keratin and low levels of collagen, so pentosidine levels are relatively low.

Kittiwakes were the only species that showed a significant trend in adults. Pentosidine also increased with age in chicks, but the relationship was driven by a single outlier that had the highest level recorded in our study. Indeed, without that outlier, there was a significant increase from chicks to adults that followed accurately the trend derived among adult birds. When averaged across age groups (each year), the r^2 value increased to 0.39, implying that the technique may have potential utility for obtaining an approximate age in this species.

The puffin data was from a poor year when most nests failed; consequently, our sample sizes were small and we had low statistical power. As was the case for kittiwakes, the maximum value for puffin chicks was higher than the maximum value for adults, which challenges the idea that pentosidine increases regularly with age. Nonetheless, our values may be biased because those individuals that were starving or that had starved (and were therefore not sampled) might have had low glucose and therefore less AGEs (Iqbal et al. 1999a). Similarly, our sample size for razorbills was also likely too small to make general conclusions, as we were unable to catch any adults (all chicks died). For murres, we did not know the age of the chicks, but they were no > 10 days old. Interestingly, the lowest level of pentosidine was in birds

aged 6–7 years (due to high collagen, Fig. 2), and values then increased. Again, this is consistent with the idea that pentosidine is lower in birds just starting to breed compared to those breeding later.

Previous studies that found relationships between age and pentosidine used only patagium or breast tissue from necropsied or euthanized birds (Iqbal et al. 1999a; Chaney et al., 2003; Fallon et al., 2006; Cooey et al., 2010). These studies included both wild and domestic birds, and so included both presumably stressed and unstressed individuals. Thus, although food restriction (reductions in glucose and other nutrition) is known to alter pentosidine expression in birds (Iqbal et al. 1997, 1999a), the differences among studies is unlikely to be explained by nutritional stress. The only other studies that obtained samples from live birds found no relationship (Le Souëf, 2012; Rattiste et al., 2015; Campion, 2015; Labbé et al., 2019). Despite high statistical power to detect an effect (e.g., 87% for kittiwakes), we did not detect such a relationship. One possibility is that glucose may react differently post-death, creating altered levels of pentosidine, thus explaining why trends were found for birds that were already dead, but not for live birds (Pozhitkov et al., 2017).

Aging in wild, long-lived birds is likely multifactorial, and there is no single compound that appears to explain a significant proportion of senescence (Ricklefs, 2008; Monaghan et al., 2008; Munshi-South and Wilkinson, 2010). For example, whereas telomere length changes consistently with age in short-lived birds, no such pattern occurs in long-lived birds (Haussmann et al., 2003; Young et al., 2013; Rattiste et al., 2015). For long-lived birds, 'biological' rather than chronological age may be important, and physiological aging may be a complicated combination of multiple factors that change with time (i.e., breeding state, glucose in diet, stress; Ricklefs, 2008; Monaghan and Haussmann, 2006). Additional work, especially longitudinally (sampling the same individual over their lifespans), could address these issues, as well as also address the issue of selective mortality. That is, birds with lower pentosidine might die young, and so the increase in pentosidine with age may be simply an artefact of a cross-sectional sampling design.

CRediT authorship contribution statement

Angelika A. Aleksieva:Investigation.**Jason R. Treberg**:Supervision.**Antony W. Diamond**:Project administration.**Scott A. Hatch**:Project administration.**Kyle H. Elliott**:Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exger.2020.110833.

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