Environmental Toxicology and Chemistry, 2025, **44(11)**, 3078–3085
https://doi.org/10.1093/etojnl/vgaf188
Advance access publication: July 25, 2025
Brief Communication

Wear influences mercury concentrations and stable isotope values in penguin feathers

L. Johnson[1], M. Connan[2], P. Bustamante[3], N. Parsons[4], S. Rutgers[5], P.G. Ryan[1,*]

[1]FitzPatrick Institute of African Ornithology, DST-NRF Centre of Excellence, University of Cape Town, Rondebosch, South Africa
[2]Department of Zoology, Marine Apex Predator Research Unit (MAPRU), Institute for Coastal and Marine Research, Nelson Mandela University, Gqeberha, South Africa
[3]Littoral Environnement et Sociétés (LIENSs), UMR 7266 CNRS-Université de la Rochelle, La Rochelle, France
[4]South African Foundation for the Conservation of Coastal Birds, Cape Town, South Africa
[5]Two Oceans Aquarium, Cape Town, South Africa

*Corresponding author: Peter G. Ryan. Email: pryan31@gmail.com

Abstract

Stable isotope (SI) values and mercury (Hg) concentrations in animal tissues are used to study migration patterns, investigate trophic ecology, and assess metal contamination. In avian studies, feathers are frequently used because they are assumed to be metabolically inert once fully grown, so their SI values and Hg concentrations remain unchanged over time, allowing investigation of retrospective information. We tested whether feather wear changes SI values ($\delta^{13}C$, $\delta^{15}N$) and Hg concentrations in penguin feathers. Newly grown (new) and recently molted (old) black back and white breast feathers from the same molt cycle were collected from captive African *Spheniscus demersus* and northern rockhopper *Eudyptes moseleyi* penguins. In both species, old feathers had a larger proportion of their barbs lacking barbules and reflected more light than new feathers, irrespective of feather color. Mercury concentrations were consistently lower in old than in new feathers in both species, decreasing by up to one third of its original content. This is likely because the highly synchronized feather growth in penguins results in Hg concentration being greatest in the feather tips, which are subject to the greatest wear. Among SI data, $\delta^{13}C$ of black feathers tended to increase as feathers aged, whereas those of white feathers decreased with age. The $\delta^{15}N$ increased as feathers aged in both black and white feathers. Such subtle changes in feather SI could affect inferences about molt location or trophic ecology. Our findings challenge the assumption that SI values and Hg concentrations, and potentially other biological markers, are fixed in inert tissues, such as feathers.

Keywords: biological markers, inert tissues, feather microstructure, feather color

Introduction

Biological markers within organisms, such as stable isotopes (SIs) and chemical pollutants, are used routinely in a number of fields including ecological (Ramos & González-Solís, 2012), toxicological (Gupta, 2009), and medical (Kumar & Sarin, 2010) fields. Three of the biological markers of choice are carbon ($^{13}C/^{12}C$; $\delta^{13}C$) and nitrogen ($^{15}N/^{14}N$; $\delta^{15}N$) SIs, and mercury (Hg). Since the mid-1990s, carbon and nitrogen SIs have been used widely as natural tracers of food webs and to infer foraging location and trophic positions of an array of consumers (Ramos & González-Solís, 2012). More recently, the use of Hg concentration as an indicator of metal contamination and an animal's position in the food web has gained momentum (e.g., Ackerman et al., 2016; Furness & Camphuysen, 1997). Present in the environment as a result of natural and anthropogenic processes, Hg was first considered for its toxicity to biota, with effects at the molecular and cellular levels affecting the physiology and behavior of animals, as well as their reproduction and survival, and ultimately affecting populations dynamics (Goutte et al., 2014; Tan et al., 2009). Because Hg is incorporated into an animal's tissues mostly through its diet, and tends to accumulate in higher trophic levels through biomagnification, it became an element of choice in food web studies (e.g., Furness & Camphuysen, 1997).

Biological markers can be analyzed in animal tissues with different integration times; some tissues are consistently renewed with a more or less rapid turnover (e.g., plasma, red blood cells, liver), whereas other tissues (e.g., feathers, claws, nails, whiskers) form at a particular point in time (Dalerum & Angerbjörn, 2005). However, feathers are functionally different to claws and nails in that, whereas claws and nails grow continuously over time, feathers are metabolically inert once fully grown (Bearhop et al., 2003; Ramos & González-Solís, 2012). Thus, feathers are assumed to store information about the bird's environment at the time of feather growth, which may differ from that experienced at the time of capture (e.g., Cherel et al., 2000). All birds must replace their feathers regularly because of wear due to the day-to-day exposure to the elements (e.g., sun, rain, physical abrasion) as well as attack from bacteria, fungi, and ectoparasites such as feather mites (Terrill & Shultz, 2023). The pattern of molt is diverse

Received: April 9, 2025. **Revised:** July 14, 2025. **Accepted:** July 16, 2025

among birds, with its timing depending on a trade-off between energy-consuming activities; feathers can thus be several months old when sampled and exhibit different degrees of wear (Jenni & Winkler, 2020).

To date, only one study has assessed the impact of wear on SI values or contaminant concentrations (e.g., Hg) of inert tissues such as feathers. Appelquist et al. (1984) submitted guillemot feathers to a range of treatments including wear, and found that loss in Hg was small (< 10% of initial content). Here, we tested the assumption that biological markers are fixed in feathers, using captive penguins as a case study. Unlike most bird species, penguins typically undergo a catastrophic molt, replacing all their feathers annually at once while fasting ashore for a few weeks (Reilly, 1994). Due to their synchronous molting process, all feathers are the same age, forming a single cohort grown over a short period. This reduces the intra-individual variability in feather composition, which is often higher in species with an asynchronous feather replacement, such as flying birds (Carravieri et al., 2014). Using captive penguins allowed us to sample feathers of known age from the same growth cohort, both newly grown and those about to be molted, from the same individuals, further reducing inter-individual variability. We tested whether feather carbon and nitrogen SI composition and Hg concentration vary as feathers age. Feather microstructure, color, carbon and nitrogen SI compositions, and Hg concentration were determined from newly grown (t_{new}) and old (t_{old}) feathers from the same growth cohort. First, we confirmed that feather microstructure is damaged as feathers age and wear. Second, we tested whether the loss of melanosomes during feather wear affects black and white feather SI composition differently, changing the melanin content and thus SI composition in black feathers but not in white feathers (Michalik et al., 2010). Third, because Hg strongly binds to keratin and is deposited in feathers during feather growth (Crewther et al., 1965), we tested whether microstructure damages during feather wear results in the loss of Hg, with no difference expected linked to feather color.

Methods

Feather collection

Long-term captive penguins housed at two facilities in Cape Town, South Africa, were used for the study: eight African penguins (APs, *Spheniscus demersus*; seven from the Southern African Foundation for the Conservation of Coastal Birds [SANCCOB], one from Two Oceans Aquarium) and eight northern rockhopper penguins (NRPs, *Eudyptes moseleyi*; all from the Two Oceans Aquarium). At the Two Oceans Aquarium, APs are kept indoors and are thus seldom exposed to sunlight, whereas NRPs are sometimes exposed to sunlight. The SANCCOB birds are in outdoor pens only partially protected by shade cloth. Prior to and during molt, APs at SANCCOB were mostly fed Indian oil sardine (*Sardinella longiceps*) from Vietnam, before their diet switched to South African sardine (*Sardinops sagax*) after the molt. At the Two Oceans Aquarium, the NRPs were fed consistently with a mix of Patagonian squid (*Doryteuthis gahi*), vannamei prawn (*Litopenaeus vannamei*) and Indian oil sardine, whereas the AP was fed South African sardine. Immediately after molting (AP: December 2015–January 2016; NRP: March–June 2016), white breast and black back feathers were clipped from each penguin (new feathers; t_{new}). The feathers were cut near the sheath rather than plucked to prevent potential regrowth (Watson, 1963). Each penguin was then resampled approximately 11 months later during their next molt, when old breast and old back feathers from the same feather cohort were collected (old feathers; t_{old}).

Assessment of feather wear

Feather integrity was assessed by examining both microstructural features and change in feather color (D'Alba et al., 2014). Feathers comprise a central shaft, the rachis, to which are attached barbs, and attached to each barb are smaller barbules often interlocking (see online supplementary material Figure S1A). Differences in feather microstructure due to wear were assessed by comparing the proportion of feather barbs with barbules at the distal end of the feathers. Feathers were photographed under a dissecting microscope (Nikon Stereoscopic Zoom Microscope SMZ1500, 10×) and ImageJ2 (Rueden et al., 2017) was used to count the number of barbs on a 5 mm transect along the rachis of each feather (see online supplementary material Methods and Figure S1A).

To test for color changes in feathers as they wear, reflectance measurements were compared between t_{new} and t_{old} feathers. Reflectance is the proportion of incident light reflected by an object; it is linked to its inherent color and is independent of the receiver and light conditions (Andersson & Prager, 2006; Endler, 1990). Reflectance measurements were taken from three black feathers and three white feathers from each penguin collected at t_{new} and t_{old} against a background of black felt material. Reflectance was measured using an Ocean Optics Jaz spectrophotometer (Ocean Optics), coupled with an optic fiber and a miniature pulsed xenon light source for ultraviolet-visible (UV-VIS; 220–750 nm) PX2 (Ocean Optics; see online supplementary material Methods). Reflectance values were analyzed in R using the *PAVO* package (R version 3.4.0; Maia et al., 2013). The spectral data averaged from the six points measured on each feather was plotted to observe the reflectance curves and to extract the hue H1 value (wavelength of maximum reflectance) for each feather type (t_{new} vs. t_{old} feathers, for black and white feathers) from each penguin species.

SI analysis

Three white and three black feathers were analyzed individually per penguin at both t_{new} and t_{old}. Feathers were washed for 2 min in chloroform-methanol (2:1) and then rinsed in successive baths of methanol and deionized water before being dried at 50 °C for 24 hr. The relative isotopic abundance of $\delta^{13}C$ and $\delta^{15}N$ was determined for homogenized feather samples at the Stable Isotope Unit, Department of Archaeology, University of Cape Town (see online supplementary material Methods). The results are presented in the usual δ notation, relative to Vienna Pee Dee Belemnite for $\delta^{13}C$ and atmospheric N_2 for $\delta^{15}N$.

Hg analysis

Mercury analysis was conducted on one white and one black feather from t_{new} and t_{old} for each penguin, except for one African penguin where old white feathers were not available. Intra-individual variation in body feather Hg is low in penguins (Carravieri et al., 2014). Prior to the Hg analysis, feathers were washed using the same procedure as for the SI analysis. They were then finely cut into a powder using stainless steel scissors. Mercury was analyzed on feather homogenates (0.6–1.5 mg), on several replicates until the relative standard deviation between measurements reached < 10%, using an Advanced Mercury Analyzer (AMA 254, Altech; see online supplementary material Methods). Mercury concentrations are expressed as $\mu g \cdot g^{-1}$ dry weight.

Data analyses

Differences in the parameter tested (barb lengths with barbules, hues, $\delta^{13}C$, $\delta^{15}N$, Hg concentration) both between black and white feathers and between t_{new} and t_{old} feathers were tested within group with paired *t*-tests (paired by individual birds) once normality of the difference was verified (Shapiro-Wilk test). When data were not normally distributed, Wilcoxon signed-rank tests were used. To account for multiple comparisons, *p*-values were adjusted using the Benjamini-Hochberg false discovery rate method (Benjamini & Hochberg, 1995). Facilities strongly influenced Hg data, with feathers from the sole AP housed at the Two Oceans Aquarium containing four to five times less Hg than the APs housed at SANCCOB. This difference was likely due to the different diets at the two facilities, so statistical comparisons for Hg were restricted to AP housed at SANCCOB and NRP housed at the Two Ocean Aquarium. Within-group comparisons of reflectance curves between t_{new} and t_{old} were performed visually based on the average values and 95% confidence intervals estimated with the *PAVO* package (Maia et al., 2013). All statistical analyses were performed in R Ver. 4.0.2 (R Core Team, 2024). The significance level (α) was set at 0.05.

Results

Feather microstructure and color analysis

Overall, between 81% ± 17% and 100% of the barb length had barbules on average in t_{new} feathers, and between 59% ± 28% and 79% ± 15% in t_{old} feathers (Figure 1A). White feathers tended to have more densely packed barbules than their black counterparts within species and age group but the differences were not significant (Table 1; Figure 1A). Old feathers tended to exhibit a larger proportion of their barb lengths without barbules compared with new feathers in both species and for both feather colors (Figure 1A). However, only old black feathers of NRPs and old white feathers of APs had significantly more of their barb length lacking barbules than t_{new} feathers (Table 1). In the other two groups, the differences were marginally not significant (Table 1).

As expected, white feathers of both penguin species exhibited higher reflectance values than black feathers (see online supplementary material Figure S2). In APs, old feathers exhibited higher reflectance values than new feathers from about 600 nm in black feathers and 450 nm in white feathers (see online supplementary material Figure S2). The differences in reflectance curves between new and old black feathers of NRPs were less marked than in APs, and there was no difference in white feathers in this species (see

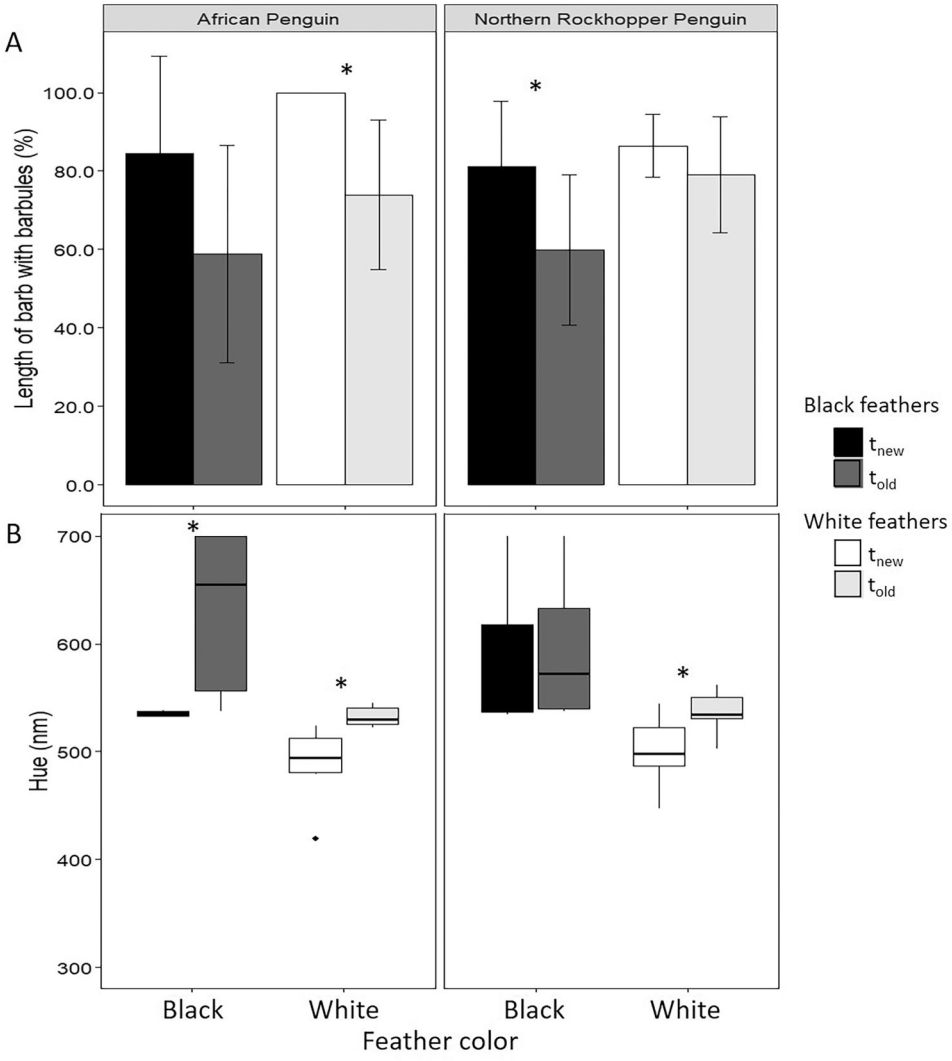

Figure 1. (A) Proportion of individual feather barbs which had barbules attached along a 5 mm section (error bars indicate ± 1 SD), and (B) hue values (minimum, median, maximum, interquartile range) of black and white, new (t_{new}) and old (t_{old}) feathers in African and northern rockhopper penguins. *Indicates significant difference between new and old feathers.

Table 1. Statistical outcomes of paired t-tests (or Wilcoxon signed-rank tests) for the comparisons between white and black feathers and between new and old feathers within penguin species.

	White vs. black feathers				New vs. old feathers			
	Old		New		White		Black	
	AP	NRP	AP	NRP	AP	NRP	AP	NRP
Barbules	t = –1.766 df = 4 *p* = 0.977	t = –2.446 df = 6 *p* = 0.977	W = 0 N_{pairs} = 5 *p* = 0.977	t = –1.007 df = 6 *p* = 0.977	**t = 3.061** **df = 4** ***p* = 0.049**	t = 1.807 df = 6 *p* = 0.071	t = 1.687 df = 6 *p* = 0.071	**t = 2.459** **df = 6** ***p* = 0.049**
Hue H1	**t = 3.214** **df = 5** ***p* = 0.012**	**W = 36** **N_{pairs} = 8** ***p* = 0.010**	**t = 3.177** **df = 5** ***p* = 0.012**	**t = 3.521** **df = 7** ***p* = 0.010**	**t = –3.169** **df = 5** ***p* = 0.033**	**t = –2.613** **df = 7** ***p* = 0.046**	**t = –2.917** **df = 5** ***p* = 0.033**	t = –0.302 df = 7 *p* = 0.772
$\delta^{13}C$	**t = –6.304** **df = 7** ***p* < 0.001**	**t = –3.386** **df = 7** ***p* = 0.006**	**t = –8.939** **df = 7** ***p* < 0.001**	**t = –8.216** **df = 7** ***p* < 0.001**	**t = 2.844** **df = 7** ***p* = 0.015**	**t = 2.983** **df = 7** ***p* = 0.015**	**t = –2.697** **df = 7** ***p* = 0.015**	**t = –2.700** **df = 7** ***p* = 0.015**
$\delta^{15}N$	t = –1.725 df = 7 *p* = 0.064	**t = –2.667** **df = 7** ***p* = 0.032**	**t = –2.219** **df = 7** ***p* = 0.041**	**t = –5.652** **df = 7** ***p* = 0.002**	t = –1.329 df = 7 *p* = 0.113	t = –2.339 df = 7 *p* = 0.052	t = –1.407 df = 7 *p* = 0.113	t = –2.572 df = 7 *p* = 0.052
Hg[a]	t = –1.148 df = 4 *p* = 0.992	t = 0.089 df = 7 *p* = 0.992	t = –1.529 df = 4 *p* = 0.992	t = –3.118 df = 7 *p* = 0.992	**t = 6.121** **df = 4** ***p* = 0.002**	**t = 4.919** **df = 7** ***p* = 0.002**	**t = 6.918** **df = 5** ***p* = 0.002**	t = 1.560 df = 7 *p* = 0.081

Note. Significant outcomes are shown in bold text. AP = African penguin; NRP = northern rockhopper penguin.
[a] AP01 removed from the data analysis.

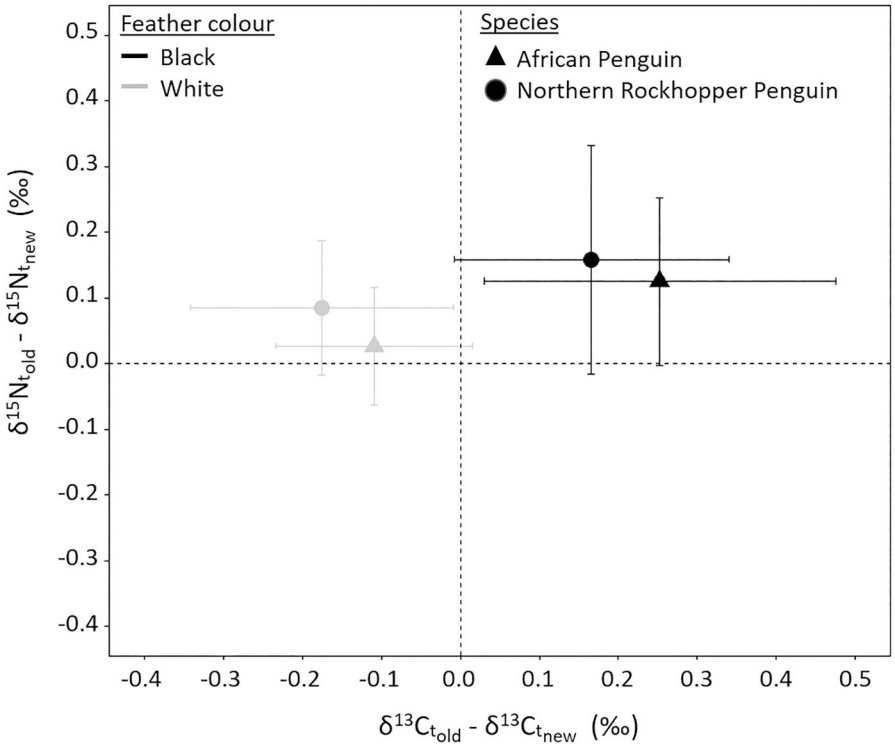

Figure 2. Mean differences in $\delta^{13}C$ and $\delta^{15}N$ values (‰) between new and old, black (A) and white (B) feathers of African (black) and northern rockhopper (grey) penguins. Error bars indicate ± 1 SD.

online supplementary material Figure S2). Hue values were between 488 ± 39 nm in white AP t_{new} feathers and 631 ± 75 nm in black AP t_{old} feathers (Figure 1B). Black feathers exhibited significantly greater hue values than white feathers in both species (Table 1; Figure 1B). When comparing t_{new} with t_{old} feathers, hue values were significantly lower in new feathers in both AP black and white feathers and in NRP white feathers (Table 1; Figure 1B).

SI composition

Carbon SI values were between –16.9 ± 0.5 ‰ in AP t_{new} black feathers and –15.7 ± 0.4 ‰ in NRP t_{new} white feathers, whereas nitrogen SI values ranged between 14.1 ± 0.6 ‰ in AP t_{new} black feathers and 14.6 ± 0.4 ‰ in NRP t_{old} white feathers (see online supplementary material Figure S3). The SI values differed between black and white feathers in both penguin species (Figure 2). Black feathers exhibited significantly lower $\delta^{13}C$ than white feathers in both new and old feathers (Table 1). Similarly, black feathers significantly exhibited lower $\delta^{15}N$ than white feathers in new feathers from both species and old NRP feathers (the difference was marginally not significant in old AP feathers; Table 1).

Significant differences in $\delta^{13}C$ values between new and old feathers were found in both penguin species. The $\delta^{13}C$ values of

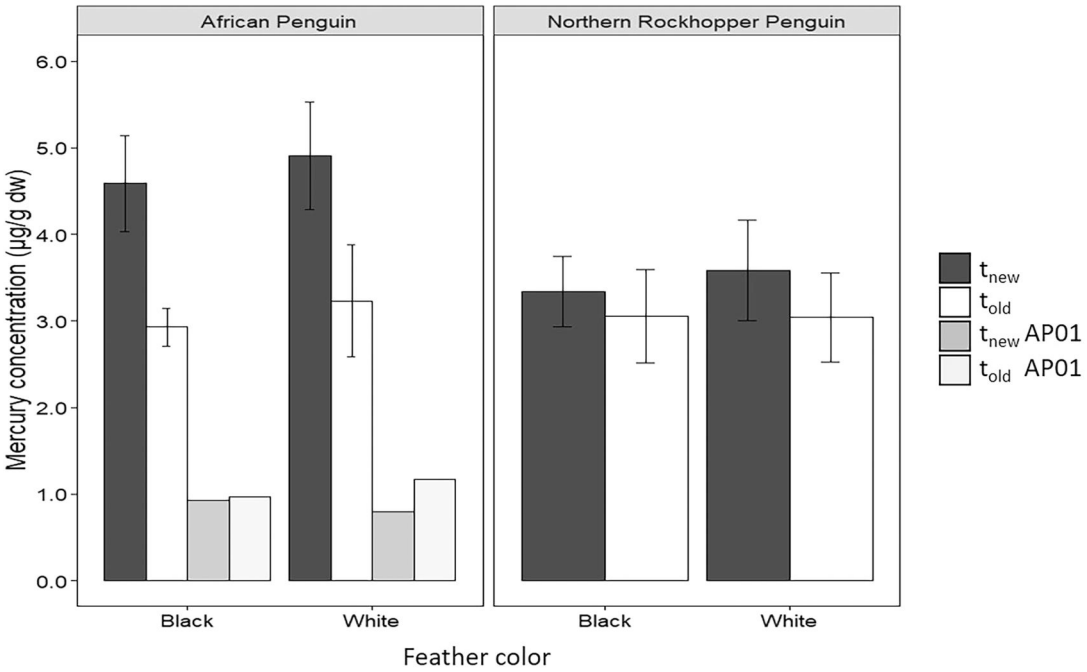

Figure 3. Mercury concentration (µg/g dry wt, mean± 1 SD) in new (t_{new}) and old (t_{old}) black and white feathers of African and northern rockhopper penguins with the African penguin from the Two Oceans Aquarium (AP01) kept separate.

black feathers increased as the feathers aged, whereas the $\delta^{13}C$ values of white feathers decreased with age (Figure 2). Old black feathers had significantly higher $\delta^{13}C$ values than new feathers in both APs (+ 0.22 ± 0.23 ‰) and NRPs (+ 0.17 ± 0.17 ‰; Table 1). By comparison, lower $\delta^{13}C$ values were measured in old white feathers than new feathers in both APs (–0.12 ± 0.12 ‰) and NRPs (–0.18 ± 0.17 ‰; Table 1). The $\delta^{15}N$ values increased as feathers aged in both black and white NRP feathers (black: + 0.16 ± 0.17 ‰, white: + 0.09 ± 0.10 ‰; Figure 2), but this increase was marginally not significant (both $p = 0.052$; Table 1) after adjusting the p-values with the false discovery rate correction factor. The $\delta^{15}N$ values did not differ significantly with age in AP feathers (black: + 0.08 ± 0.16 ‰, white: + 0.05 ± 0.11 ‰; Table 1, Figure 2).

Hg concentration

Both extreme Hg concentrations were found in APs, from 2.93 ± 0.22 µg/g dry weight in t_{old} black feathers to 4.91 ± 0.62 µg/g dry weight in t_{new} white feathers (see online supplementary material Figure S4). Feather color had no effect on Hg concentrations among feathers of the same age within each species (Table 1, Figure 3). Old white feathers were significantly depleted in Hg compared with new feathers in both species (Table 1). Old white feathers contained 34.0% ± 11.8% and 16.3% ± 7.5% less Hg than new feathers for AP and NRP, respectively. A similar trend was observed in black AP feathers, with old feathers containing 35.4% ± 9.0% less Hg than new feathers (Table 1, Figure 3). Old black NRP feathers also tended to contained less Hg than new feathers, but the difference was not significant (Table 1, Figure 3).

Discussion

Using captive APs and NRPs as a case study, we show that the SI composition and Hg concentrations in feathers can change over time. Although changes in $\delta^{13}C$ and $\delta^{15}N$ were subtle (averaging 0.1 to 0.2 ‰, and within a similar magnitude to the measurement errors), those for Hg were more important, with up to 35% less Hg measured in old versus new black AP feathers. These results raise concerns about simply assuming that all components in feathers remain the same once growth is complete. We compared feathers from the same cohort of feathers, not exactly the same feather. However, our results are compelling because the catastrophic molt in penguins results in all feathers being grown at the same time, and because the same trend (even when subtle as for SIs) was found in multiple birds. Therefore, we are confident that $\delta^{13}C$, $\delta^{15}N$, and Hg concentrations can change in feathers due to wear.

Differences in SIs can occur within the same feather. Such differences can result from the source of amino acids used to synthesize feather keratin. For example, king penguin (*Aptenodytes patagonicus*) feathers are synthesized from both stored nutrients and nutrients derived from recent diet; the tips of feathers, which are grown first, use amino acids from at-sea diet and are enriched in ^{13}C compared with the feather base, which is synthesized from stored amino acids (Cherel et al., 2005). In flying seabirds, diet switching during molt can also result in differences in SI values within a single feather; for example, newer sections of great skua (*Catharacta skua*) feathers had SI values that matched the most recent diet (Bearhop et al., 2002). Similarly, Hg concentrations within and among feathers of the same individuals can change as the Hg body pool depletes, when Hg gets deposited in feathers as they grow (Gatt et al., 2021). However, neither of these studies showed that feather components can change after feathers have finished growing.

When assessing possible explanations for changes in SI values between new and old feathers, color was the most logical factor to consider. Because melanin is synthesized from the amino acid tyrosine, which is naturally depleted in ^{13}C and ^{15}N (McCullagh et al., 2005), black feathers typically exhibit lower $\delta^{13}C$ and $\delta^{15}N$ values than white feathers (Michalik et al., 2010), as was consistently the case in our study. Old black feathers from both penguin species appeared browner and had a higher reflectance

than new black feathers, suggesting a change in melanin content as feathers age. Although we did not measure melanin content directly, total melanin content in feathers is a strong predictor of feather hue, saturation, and brightness, which are used for the calculation of reflectance; increasing melanin content results in increased color scores (McGraw et al., 2005). The lower color scores we observed in old black penguin feathers therefore suggest reduced melanin content.

Melanin is enclosed in granular melanosomes (Hill & McGraw, 2006), which make black feathers stronger and more wear-resistant than white feathers (Bonser, 1995). If melanin content changed as feathers age, then it would likely be because of feather wear due to the loss of melanin-containing barbules (Bonser, 1995). Barbule density of feathers has a larger effect on feather color variation than melanosome density (D'Alba et al., 2014). Feather microstructure observed in both penguin species indicated that old feathers had fewer barbules at the distal end of the feathers than new feathers. As feathers age they are worn by physical abrasion (typically from airborne particles but also collision with objects, repeated bending, and overextension), exposure to UV radiation in sunlight, and attacks by bacteria, fungi, and feather lice (Burtt, 1986; Jenni & Winkler, 2020). Therefore, the physical wear of penguin feathers resulted in the loss of melanosomes (and thus melanin) in old black feathers compared with newly grown feathers, increasing $\delta^{13}C$ and $\delta^{15}N$ values, as observed in our study. A nanostructure analysis of cross sections of newly grown and worn feathers could be used to estimate the physical loss of melanosomes (D'Alba et al., 2014).

The loss of Hg as feathers age is also likely linked to feather wear. As birds molt, circulating Hg binds to keratin and is deposited in feathers. This decreases the Hg whole body content (if Hg in the diet is limited), and thus the distal ends of feathers, which are grown first, tend to exhibit higher Hg contents than feather bases, which are grown last. This is particularly important in large feathers like the primaries (Furness, 1993) but is also likely to result in strong gradients in penguins, where growth of all feathers is highly synchronized. Wear of the feather tips thus releases more Hg, reducing the concentration of Hg in old feathers. Appelquist et al. (1984) did not find marked differences in Hg concentrations between new feathers and feathers that were artificially weathered for 2–8 months, but they analyzed the shaft at the base of the web, which is unlikely to be affected by wear. Interestingly, the greatest Hg losses observed in our study occurred in APs, where the highest losses of barbules were also detected.

We explored the impact of mechanical damage of the feather on the color of the feather (i.e., the physical loss of barbules and thus melanosomes). We did not assess whether there was any chemical break down of melanin (Ginn & Melville, 1983), which could potentially influence the SI values of the feathers. Combining the results we obtained with biochemical analyses such as melanin content and/or amino acid composition would be the next logical step to deepen our understanding on the influence of feather wear on SI values. Fine biochemical analyses comparing barb and barbule compositions may also shed light into the changes observed in the black but also white feathers. Melanin content cannot be the sole factor influencing the SI values in feathers, as changes were also detected between new and old white feathers. Furthermore, the difference in color between new and old feathers was higher in APs, but the difference in SI values was most marked in NRP feathers, which suggests that another phenomenon may intervene in addition to the change in color.

Although we found differences in $\delta^{13}C$, $\delta^{15}N$, and Hg concentrations between new and old penguin feathers, they may not accurately reflect changes in wild birds. Captive penguins are not exposed to the same level of sun exposure as wild birds, which reduces wear from UV exposure. This difference in sun exposure may also explain the differences between the two penguin species studied. The NRPs were mostly housed indoors, out of direct sunlight and showed less color fading than APs, which were housed outdoors. On the other hand, the plumage of captive penguins can experience greater mechanical wear due to physical abrasion with enclosure walls and floors. To estimate to which extent wear differs between captive and wild penguins, wear estimates would need to be conducted on new and molting feathers from wild penguins. The challenge here would be capturing the same individuals in successive years.

Our study suggests that elemental components are not always fixed in penguin feathers and that wear may explain, at least in part, the changes between new and old feathers. It should be noted, however, that in the absence of more detailed information on melanin content of feathers, the mechanisms linking feather wear and SI values remain unexplored. The catastrophic annual molt of penguins gives the opportunity to sample feathers from two growth generations at once: 1-year-old feathers together with newly grown feathers. This opportunity has been used in several studies (e.g., Jaeger & Cherel, 2011; Whitehead et al., 2017) and differences were interpreted as environment differences between years. Our work suggests that the differences observed may result from both changes in the environment and from intrinsic changes in the composition of feathers. Due to the limited sample size, we did not investigate whether feather wear differs between sexes and how this may influence elemental composition. However, given the physiological and, depending on the species, behavioral differences between male and female penguins, this warrants further investigation. Further work should also expand this research to flying wild birds, where feather wear influences feather color (Osborne & Ryan, 2021). Future studies could explore intra-individual variability (e.g., differences in wear of various feather types within the same individual with asynchronous molt) or investigate the effects of environmental exposure factors (e.g., UV radiation, physical abrasion).

To conclude, we recommend that researchers using feathers as a tissue for biomarker analyses should note the timing of molt in the studied species and report the degree of wear of feathers analyzed. This will allow wear to be considered as a factor in the study as well as inform any subsequent studies that use the results. For example, when comparing different seabird colonies, it would be important to ensure that feather condition does not contribute to spatial variations in biological markers. The same consideration applies to temporal studies. In addition, plumage color is also affected in museum specimens (Doucet & Hill, 2009), which needs to be considered when making ecological inferences about historic changes in birds' environment or diet based on feathers from museum specimens (e.g., Hilton et al., 2006).

Supplementary material

Supplementary material is available online at *Environmental Toxicology and Chemistry*.

Data availability

Data are available from the corresponding author on reasonable request.

Author contributions

Laurie Johnson (Formal analysis, Investigation, Writing—original draft), Maelle Connan (Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing—original draft), Paco Bustamante (Conceptualization, Funding acquisition, Methodology, Resources, Writing—review & editing), Nola Parsons (Investigation, Resources, Writing—review & editing), Shanet Rutgers (Investigation, Resources, Writing—review & editing), and Peter Ryan (Conceptualization, Methodology, Resources, Supervision, Writing—review & editing)

Funding

This research was funded by the National Research Foundation of South Africa and the Contrat de Projet État-Région and the Fonds Européen de Développement Régional that funded the Advanced Mercury Analyzer of LIENSs laboratory. The University of Cape Town and the Ada & Bertie Levenstein Bursary award provided funding to L.J. for her MSc thesis.

Conflicts of interest

None declared.

Ethical statement

Animal ethics clearance was granted from the University of Cape Town Science Faculty Animal Ethics Committee (ethics number: 2015/v17/PR).

Acknowledgments

We thank P. Muller for her assistance with photographing the feather samples and A. Coetzee for advising on feather color analysis. The authors are indebted to I. Newton and J. Lanham from the University of Cape Town for timeously running the stable isotope analyses. The authors thank M. Brault-Favrou from the Plateforme Analyses Élémentaires of LIENSs laboratory (La Rochelle University) for her support during Hg analyses. P.B. is an honorary member of the IUF (Institut Universitaire de France). We thank the Editor and the two reviewers for their detailed comments, which greatly improved the manuscript.

References

Ackerman, J. T., Eagles-Smith, C. A., Herzog, M. P., Hartman, C. A., Peterson, S. H., Evers, D. C., Jackson, A. K., Elliott, J. E., Vander Pol, S. S., & Bryan, C. E. (2016). Avian mercury exposure and toxicological risk across western North America: A synthesis. *Science of the Total Environment*, *568*, 749–769. https://doi.org/10.1016/j.scitotenv.2016.03.071.

Andersson, S., & Prager, M. (2006). Quantifying colours. In G. E. Hill & K. J. McGraw, (Eds.), *Bird coloration volume 1: Mechanisms and measurements* (pp. 90–147). Harvard University Press.

Appelquist, H., Asbirk, S., & Drabæk, I. (1984). Mercury monitoring: Mercury stability in bird feathers. *Marine Pollution Bulletin*, *15*, 22–24. https://doi.org/10.1016/0025-326X(84)90419-3.

Bearhop, S., Waldron, S., Votier, S. C., & Furness, R. W. (2002). Factors that influence assimilation rates and fractionation of nitrogen and carbon stable isotopes in avian blood and feathers. *Physiological and Biochemical Zoology*, *75*, 451–458. https://doi.org/10.1086/342800.

Bearhop, S., Furness, R. W., Hilton, G. M., Votier, S. C., & Waldron, S. (2003). A forensic approach to understanding diet and habitat use from stable isotope analysis of (avian) claw material. *Functional Ecology*, *17*, 270–275. https://www.jstor.org/stable/3599183

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society, Series B (Methodological)*, *57*, 289–300.

Bonser, R. H. C. (1995). Melanin and the abrasion resistance of feathers. *Condor*, *97*, 590–591.

Burtt, E. H. J. (1986). An analysis of physical, physiological, and optical aspects of avian colouration with emphasis on wood-warblers. *Ornithological Monographs*, *38*, iii–126. https://doi.org/10.2307/40166782

Carravieri, A., Bustamante, P., Churlaud, C., Fromant, A., & Cherel, Y. (2014). Molting patterns drive within-individual variations of stable isotopes and mercury in seabird body feathers: Implications for monitoring of the marine environment. *Marine Biology*, *161*, 963–968. https://doi.org/10.1007/s00227-014-2394-x.

Cherel, Y., Hobson, K. A., & Weimerskirch, H. (2000). Using stable-isotope analysis of feathers to distinguish molting and breeding origins of seabirds. *Oecologia*, *122*, 155–162. https://doi.org/10.1007/PL00008843

Cherel, Y., Hobson, K. A., Bailleul, F., & Groscolas, R. (2005). Nutrition, physiology, and stable isotopes: New information from fasting and molting penguins. *Ecology*, *86*, 2881–2888. https://doi.org/10.1890/05-0562.

Crewther, W., Fraser, R., Lennox, F., & Lindley, H. (1965). The chemistry of keratins. *Advances in Protein Chemistry*, *20*, 191–346. https://doi.org/10.1016/S0065-3233(08)60390-3.

D'Alba, L., van Hemert, C., Spencer, K. A., Heidinger, B. J., Gill, L., Evans, N. P., Monaghan, P., Handel, C. M., & Shawkey, M. D. (2014). Melanin-based color of plumage: Role of condition and of feathers' "microstructure." *Integrative and Comparative Biology*, 54, 633–644. https://doi.org/10.1093/icb/icu094.

Dalerum, F., & Angerbjörn, A. (2005). Resolving temporal variation in vertebrate diets using naturally occurring stable isotopes. *Oecologia*, 144, 647–658. https://doi.org/10.1007/s00442-005-0118-0.

Doucet, S. M., & Hill, G. E. (2009). Do museum specimens accurately represent wild birds? A case study of carotenoid, melanin, and structural colours in long-tailed manakins *Chiroxiphia linearis*. *Journal of Avian Biology*, 40, 146–156. https://doi.org/10.1111/j.1600-048X.2009.03763.x.

Endler, J. A. (1990). On the measurement and classification of colour in studies of animal colour patterns. *Biological Journal of the Linnean Society*, *41*, 315–352. https://doi.org/10.1111/j.1095-8312.1990.tb00839.x.

Furness, R. W. (1993). Birds as monitors of pollutants. In R. W. Furness & J. J. D. Greenwood (Eds.), *Birds as monitors of environmental change* (pp. 86–143). Springer.

Furness, R. W., & Camphuysen, K. C. J. (1997). Seabirds as monitors of the marine environment. *ICES Journal of Marine Science*, 54, 726–737. https://doi.org/10.1006/jmsc.1997.0243.

Gatt, M.-C., Furtado, R., Granadeiro, J. P., Lopes, D., Pereira, E., & Catry, P. (2021). Untangling causes of variation in mercury concentration between flight feathers. *Environmental Pollution*, 269, 116105. https://doi.org/10.1016/j.envpol.2020.116105.

Ginn, H. B., & Melville, D. S. (1983). *Moult in birds*. British Trust for Ornithology.

Goutte, A., Bustamante, P., Barbraud, C., Delord, K., Weimerskirch, H., & Chastel, O. (2014). Demographic responses to mercury

exposure in two closely-related Antarctic top predators. *Ecology*, 95, 1075–1086. https://doi.org/10.1890/13-1229.1.

Gupta, R. C. (2009). *Biomarkers in toxicology*. Academic Press Elsevier.

Hill, G. E., & McGraw, K. J. (2006). *Bird coloration—mechanisms and measurements*. Harvard University Press.

Hilton, G. M., Thompson, D. R., Sagar, P. M., Cuthbert, R. J., Cherel, Y., & Bury, S. J. (2006). A stable isotopic investigation into the causes of decline in a sub-Antarctic predator, the rockhopper penguin *Eudyptes chrysocome*. *Global Change Biology*, 12, 611–625. https://doi.org/10.1111/j.1365-2486.2006.01130.x.

Jaeger, A., & Cherel, Y. (2011). Isotopic investigation of contemporary and historic changes in penguin trophic niches and carrying capacity of the Southern Indian Ocean. *PLoS One*, 6, e16484. https://doi.org/10.1371/journal.pone.0016484.

Jenni, L., & Winkler, R. (2020). *The biology of moult in birds*. Bloomsbury Publishing.

Kumar, M., & Sarin, S. K. (2010). Biomarkers of diseases in medicine. *Current Trends in Sciences*, 15, 403–417.

Maia, R., Eliason, C. M., Bitton, P.-P., Doucet, S. M., & Shawkey, M. D. (2013). pavo: an R package for the analysis, visualization and organization of spectral data. *Methods in Ecology and Evolution*, 4, 906–913. https://doi.org/10.1111/2041-210X.12069.

McCullagh, J. S., Tripp, J. A., & Hedges, R. E. (2005). Carbon isotope analysis of bulk keratin and single amino acids from British and North American hair. *Rapid Communications in Mass Spectrometry*, 19, 3227–3231. https://doi.org/10.1002/rcm.2150.

McGraw, K. J., Safran, R. J., & Wakamatsu, K. (2005). How feather colour reflects its melanin content. *Functional Ecology*, 19, 816–821. https://doi.org/10.1111/j.1365-2435.2005.01032.x.

Michalik, A., McGill, R. A. R., Furness, R. W., Eggers, T., van Noordwijk, H. J., & Quillfeldt, P. (2010). Black and white—does melanin change the bulk carbon and nitrogen isotope values of feathers? *Rapid Communications in Mass Spectrometry: RCM*, 24, 875–878. https://doi.org/10.1002/rcm.4462.

Osborne, A., & Ryan, P. G. (2021). Using digital photography to study moult extent in breeding seabirds. *Ostrich*, 92, 225–228. https://doi.org/10.2989/00306525.2021.1897699.

R Core Team. (2024). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing.

Ramos, R., & González-Solís, J. (2012). Trace me if you can: The use of intrinsic biogeochemical markers in marine top predators. *Frontiers in Ecology and the Environment*, 10, 258–266. https://doi.org/10.1890/110140.

Reilly, P. N. (1994). *Penguins of the world*. Oxford University Press.

Rueden, C. T., Schindelin, J., Hiner, M. C., DeZonia, B. E., Walter, A. E., Arena, E. T., & Eliceiri, K. W. (2017). ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics*, 18, 529. https://doi.org/10.1186/s12859-017-1934-z.

Tan, S. W., Meiller, J. C., & Mahaffey, K. R. (2009). The endocrine effects of mercury in humans and wildlife. *Critical Reviews in Toxicology*, 39, 228–269. https://doi.org/10.1080/10408440802233259.

Terrill, R. S., & Shultz, A. J. (2023). Feather function and the evolution of birds. *Biological Reviews of the Cambridge Philosophical Society*, 98, 540–566. https://doi.org/10.1111/brv.12918.

Watson, G. E. (1963). The mechanism of feather replacement during natural molt. *The Auk*, 80, 486–495. https://doi.org/10.2307/4082854.

Whitehead, T. O., Connan, M., Ropert-Coudert, Y., & Ryan, P. G. (2017). Subtle but significant segregation in the feeding ecology of sympatric penguins during the critical pre-moult period. *Marine Ecology Progress Series*, 565, 227–236. https://doi.org/10.3354/meps12017.

Environmental Toxicology and Chemistry, 2025, 44, 3078–3085
https://doi.org/10.1093/etojnl/vgaf188
Brief Communication