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Sex- and age-related variation in metal content of penguin feathers

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Abstract

The presence of xenobiotics, such as metals, in ecosystems is concerning due to their durability and they pose a threat to the health and life of organisms. Moreover, mercury can biomagnify in many marine food chains and, therefore, organisms at higher trophic levels can be adversely impacted. Although feathers have been used extensively as a bio-monitoring tool, only a few studies have addressed the effect of both age and sex on metal accumulation. In this study, the concentrations of trace elements were determined in the feathers of all members of a captive colony of African Penguins (*Spheniscus demersus*) housed in a zoological facility in Italy. Tests were performed by Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) to detect aluminum, arsenic, cadmium, cobalt, chromium, copper, iron, manganese, nickel, lead, selenium, tin, vanadium, and zinc. Mercury was detected by a Direct Mercury Analyzer. Sexing was performed by a molecular approach based on analyzing the chromo-helicase-DNA-binding1 (CHD1) gene, located on the sex chromosomes. Sex- and age-related differences were studied in order to investigate the different patterns of metal bioaccumulation between male and female individuals and between adults and juveniles. Juvenile females had significantly higher arsenic levels than males, while selenium levels increased significantly with age in both sexes. Penguins kept in controlled environments- given that diet and habitat are under strict control- represent a unique opportunity to determine if and how metal bioaccumulation is related to sex and age.

Keywords: African penguin, seabirds, *Spheniscus*, trace elements.

1. Introduction

Over the last decade, there has been an increasing interest in the use of sentinel organisms for pollution monitoring studies (Burger et al. 2008). Of these, birds have received particular attention, because they are exposed to heavy metals, both through environmental exposure and diet (Roux and Marra 2007). While many host factors, such as size and age, have received considerable attention in metal bioaccumulation studies in seabirds, gender has received relatively little attention (Burger et al., 2003). Adults of larger species, which have longer lifespans and are at higher trophic position were found to accumulate higher levels of mercury (Hg) in their feathers, due to biomagnification phenomena through the food chain (Burger and Gochfeld, 2000; Carravieri et al., 2013). Moreover, in long-living seabirds, adults have higher metal content in their feathers than chicks, as the time interval available for accumulation is longer in adults (Burger and Gochfeld, 2000; Bond and Diamond, 2009b). However, there are sex-based differences in the excretion of contaminants and sex-related differences in the fate and effects of chemicals in seabirds; sex can affect exposure and susceptibility to contaminants and influences the ability to rid the body of these pollutants (Burger et al., 2007). Most excretion methods are similar for both males and females, but females can also excrete contaminants in their eggs and embryos (Burger et al. 2003).

Bird’s feathers are useful non-invasive indicators of metal contamination, as a relatively high proportion of the body burden of certain metals, such as mercury, is stored in the feathers, because of their affinity to –SH groups of keratin. Moreover, several studies have shown that a high correlation exists between levels of certain contaminants in the diet of birds and relative levels detected in their feathers (e.g. mercury, Becker et al. 2002; Brasso et al. 2014). Another advantage of using feathers in such analyses is that they can be easily collected and, if necessary, repeatedly sampled without affecting the health and condition of the individuals being studied (Adout et al. 2007). However, in many studies using bird feathers as an indication of internal body burdens, the sex of the different individuals is not determined (Burger and Gochfeld, 2000; Dauwe et al. 2002; Markowski et al. 2013; Ansara-Ross et al. 2013). Accordingly, many bird species (including penguins) are not obviously sexually dimorphic and sex determination is therefore impossible in the field. In the very few studies considering sexual differences, a limited number of elements were investigated. Dauwe et al. (2002) examined cadmium (Cd), lead (Pb), copper (Cu) and zinc (Zn) levels in great and blue tits, considering both age and gender, and they found that only the Zn concentration was significantly higher in males than in females. Lucia et al. (2010) investigated the presence of aluminum (Al), arsenic (As), Cd, Cu, Pb, Hg, nickel (Ni), selenium (Se) and Zn in feathers of aquatic birds. They found that Cd accumulation increased with age, while As was influenced by sex, as female birds displayed higher concentrations in the liver and feathers compared to male birds. Mansouri and Hoshyari (2012) investigated the levels of Ni in the feathers of the Western Reef Heron (*Egretta gularis*) and Siberian Gull (*Larus heuglini*), but no significant differences were found between gender and age groups in either species. Finally, very few studies have been designed to determine age- or gender-differences in metal content of penguin tissues. These studies have mostly focused on Hg (e.g. Frias et al. 2012; Carravieri et al. 2013) and did not consider other trace elements. Feather metal concentrations were generally found to be species-specific, and a high variance in metal levels between individuals for some species, but not for others, were registered (Burger and Gochfeld, 2000).

The African or Jackass Penguin (*Spheniscus demersus*) is a colonial seabird endemic to South Africa and Namibia. The current conservation status of this species is “endangered”, according to the Red List of Threatened Species of the IUCN (International Union for Conservation of Nature), and the wild African Penguin population has dramatically decreased in recent years to less than 75,000-80,000 mature individuals (Birdlife International, 2013). The African Penguin therefore faces a high risk of extinction, and as a result, *in-situ* conservation programs are becoming increasingly crucial. The body size of an African penguin is 45–68 cm in height and 3 kg in weight. Sexual dimorphism is not evident, but males are generally larger than females, although measurements tend to overlap. In the wild, individuals of the species tend to live from 10 to 27 years (Whittington et al. 2000) but captive birds live significantly longer. Sexual maturity is reached at 3-4 years in wild specimens, but captive penguins are observed to breed at 2-3 years. After obtaining the adult plumage, penguins molt annually, and the feather-shedding phase lasts for 12.7 ± 1.4 days (Randall et al. 1986). As breeding and molting are energetically demanding activities in the annual cycle of the adult penguin, the timing of these events should coincide with periods of favorable environmental conditions, particularly with the availability of food.

Several African penguin colonies are housed in zoos and aquaria worldwide for *ex-situ* conservation purposes. According to the International Species Information System (www.isis.org), 2394 *Spheniscus demersus* individuals live under human care (species holding data for *Spheniscus* as of September 27, 2014). The use of captive birds to monitor pollutants offers several advantages over the analysis of biotic and abiotic matrices (Falkowska et al. 2013). Of these, seabirds are considered to be among the most reliable indicators of environmental changes; with specific reference to penguins, they are particularly relevant as they occupy a high position in many marine food chains and accumulate metals and other toxic elements in their tissues at concentrations of several orders of magnitude above environmental levels (Barbieri et al. 2010). Moreover, penguins living in *ex-situ* colonies are confined to controlled areas and have a very special and homogeneous diet, usually limited to one or two species of commercially-available fish. Therefore, they constitute a unique opportunity to investigate sex- and age-related differences in trace elements using the feathers as a noninvasive bio-monitoring tool. Our aim was to assess whether sex and/or age can affect the accumulation of essential (arsenic, chromium, copper, iron, manganese, nickel, selenium and zinc) and non-essential (aluminum, cadmium, cobalt, lead, mercury, tin, vanadium) elements in a large captive colony of African penguins.

2. Methods and Materials

2.1 Ethical statement

This research conformed to the Ethical Guidelines for the Conduct of Research on Animals by Zoos and Aquariums (WAZA 2005), and was conducted with the approval of the Ethical Committee of the Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta (11168; 14 July 2014). During collection of feathers, we made every effort to minimize distress to the penguins.

2.2 Penguins and collection of samples

Feathers were collected in March 2014 from a captive colony of 46 penguins housed at the biopark Zoom Torino (Cumiana, Torino; 44°56' N, 7°25' E; www.zoomtorino.it). The colony was composed of 25 adult breeders and 21 sexually immature juveniles (< 2 years old). Penguins were maintained in an outdoor communal exhibit of 1500 m^2, which included a pond of 120 m^2 (maximum depth: 3 m). All penguins were fed with herrings (*Clupea harengus*), purchased from an animal food retailer, which had been caught in the northeast Atlantic Ocean (FAO fishing area 27).

Samples of feathers up to a total weight of 0.3 - 0.5 g were cut from the back of each penguin. The cut was made with scissors between the *calamus* and the vane of the feather, in order not to cause any pain to the bird. Finally, a blood sample (1 mL) was extracted from a vein of the foot of each bird with a needle and a vacuum plastic tube (4.9 mL) with lithium heparin. After collection, all samples were stored for subsequent laboratory analyses.

2.3 Molecular sexing

Blood samples were extracted using the Pure Link^{TM} Genomic DNA Mini Kit (Invitrogen, Grand Island, NY USA). PCR was carried out using the primers P8 and P2, previously described by Griffiths (1998). Primer P2 was labelled with Hex fluorescent dye at the 5' end, to be used with capillary gel electrophoresis. PCR was carried out in a total volume of 25 µL containing 50-60 ng of genomic DNA using HotStarTaq Qiagen (1.5 U), Buffer containing Mg^{2+} (1.5 mM), dNTPs (0.2 mM each) and the primers reported above (300 nM each). PCR was performed in a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Life Technologies Monza, Italy). An initial activation step at 95 °C for 15 min was followed by 40 cycles of 95 °C for 30 sec, 48 °C for 30 sec, and 65 °C for 1 min. A final run of 65 °C for 5 min completed the program.

Each amplification product, diluted at 1:100, was added to a mix containing Rox Size Standard (Life Technologies, Grand Island, NY USA) and formamide, and then subjected to capillary electrophoresis on an ABI 3130 Genetic Analyzer (Life Technologies, Grand Island, NY USA). The size of the amplification products was determined by GeneMapper software analysis. Males were characterized by just one peak at 364 bp, while females had two peaks at 364 and 380 bp.

2.4 Analytical methods

Surface lipids and contaminants were removed from the feathers in four cleaning steps: a Triton-x-100 (0.01%) bath (for at least 4 hours), followed by one rinse with deionized water, two successive vigorous washes with methanol, and a final rinse with deionized water. Feathers were dried at 50°C until a constant weight was obtained, and were then minced. Prior to analysis, samples were divided into two sub-samples, one for mercury quantification with a Direct Mercury Analyzer (DMA-80 Analyzer from Milestone, Shelton, CT, USA) and the other for detecting all the other metals by Inductively Coupled Plasma - Mass Spectrometry (ICP-MS Xseries II from Thermo Scientific, Bremen, Germany). The DMA-80 analyzer performed thermal decomposition, catalytic reduction, amalgamation, desorption and atomic absorption spectroscopy without having to pre-treat the samples, and with no waste generation. Between 0.05 g and 0.1 g of feathers, according to availability, were directly weighed on graphite shuttles and processed with an output result, for mercury content, in about 5 min (per sample).

Determining levels of Al, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se, Sn, V and Zn was performed after wet digestion with acids and oxidants (HNO_3 and H_2O_2) of the highest quality grade (Suprapure). In this case, between 0.05 and 0.27 g of feathers, according to availability, and 0.10 g of pooled fish were subjected to microwave digestion (microwave oven ETHOS 1 from Milestone, Shelton, CT, USA) with 7 mL of HNO_3 (70% v/v) and 1.5 mL of H_2O_2 (30% v/v). Samples were then brought to a final weight of 50 g with ultrapure water (Arium611VF system from Sartorius Stedim Italy S.p.A., Antella - Bagno a Ripoli, FI, Italy). Multi-elemental determination was performed with ICP-MS after daily optimization of instrumental parameters and using an external standard calibration curve; Rodium and Germanium were used as internal standards. Analytical performances were verified by processing Certified Reference Materials (Dogfish liver -DOLT-4 from the National Research Council of Canada, and Oyster Tissue-SRM 1566b from the National Institute of Standard and Technology), along with blank reagents in each analytical session. The recoveries for reference materials ranged from 85 to 120% for DOLT-4 and from 82 to 117% for SRM 1566b. The limit of quantitation (LOQ) was 0.010 mg Kg^{-1} for all elements, except for Hg that was 0.034 mg Kg^{-1}. The selenium:mercury molar ratio was calculated as follows: for each bird class (adult males, adult females, juvenile males, juvenile females) the mercury and selenium concentrations in ng $g-^{1}$ (wet weight) were divided by their respective atomic weights (200.59 and 789.00) to obtain the molar concentrations (nmol $g-^{1}$), which allowed us to calculate the mean molar ratios. Note that this is a ratio of the mean values.

2.5 Statistical analysis

One half of the values of the respective limit of quantitation (LOQ) were substituted for those values below the limit of quantitation and used in statistical analysis. Data were tested for normality by using the Kolmogorov-Smirnov test. Since data distribution was normal, comparisons of the mean metal concentrations between age classes were carried out using a Student's *t-test*. All analyses were performed using the SPSS version 20.0 for Macintosh.

3. Results and Discussion

3.1 Sex-related differences

Metal concentrations in birds are highly species-specific (Burger and Gochfeld, 2000). Moreover, a few gender-related differences have been found by Burger et al. (2007) in species with sexual dimorphism in body size. In particular, females usually show higher levels of metals than males, and this suggests that the mechanism of excretion into eggs and eggshells is not very effective, or that uptake is greater (Burger et al., 2007). In our study, there were no significant sex-related differences in concentrations of essential elements, with the exception of As (Figure 1a, Table 1). Arsenic is a well-known environmental contaminant, potentially toxic even at trace levels but essential for life at very low concentrations as ultra-trace element (Cox, 1995). The concentration of arsenic was significantly higher in feathers of juvenile females than in those of juvenile males (t

= -2.97, df = 19, p < 0.005; Figure 1a), while in adults there were no significant sex-related differences. Generally, arsenic uptake is via the diet in marine animals (Kubota et al. 2001) and arsenic does not biomagnified through food chains. Accordingly, as observed for other metals that are not biomagnified, lower trophic marine animals show higher arsenic concentrations than higher trophic marine animals (Rahaman et al. 2012). Studies on As levels in high-trophic-level marine organisms are scarce, and researches are now focusing on the mechanism of As accumulation, considering its possible role as an endocrine disruptor (Georgescu et al. 2011). Lucia et al. (2010) found that female birds displayed higher As concentrations in the liver and feathers than male birds. Similarly, Taggart et al. (2006) examined As, Zn, Se, Pb and Cu levels in the livers and bones of five waterfowl species from SW Spain and found higher concentrations of As in female bones and – as in our case - higher concentrations in juveniles than in adults. As arsenic is an essential element, this difference could reflect a different physiological requirement at a particular growth stage. Egg transfer is metal specific and although it is well known that mercury is effectively transferred from females to eggs in seabirds (Robinson et al., 2012), limited information is available on the maternal transfer of arsenicals to seabird eggs. Kubota and coauthors (2002) studied the maternal transfer of arsenicals to eggs of the black-tailed gull. As composition in the eggs was similar to that in tissues of the mother bird, and the percentage of As in eggs was about 11% of that of the mother.

Examining sex-related differences in response to chemicals is complicated in wild birds because of the differences in niches and forage (Burger et al. 2007), as well as the difficulties in determining the sex of non-dimorphic species. Sex and age of vertebrate top predators are known to be involved in the variation of tissue trace element concentrations (Kojadinovic et al. 2007a). Metal accumulation in birds can be largely affected by contamination of the surrounding environment (Markowski et al. 2013). Top-level piscivores, such as penguins accumulate much higher levels of contaminants than birds that are lower on the food chain (Lodenius and Solonen 2013). Very few studies consider sex differences in penguins and tend to only focus on Hg (e.g. Becker et al. 2002; Frias et al. 2012). Despite being naturally occurring, Hg is a pervasive environmental contaminant that negatively affects humans and wildlife. The diet and foraging ecology of penguins may play an important role in explaining feather Hg levels in some penguin species, because ingestion of food is the main route of Hg exposure in birds (Lodenius and Solonen 2013; Burger et al., 2014). In particular, males of the Gentoo penguin (*Pygoscelis papua*) in South Georgia were shown to have higher levels of Hg in their feathers than females (Becker et al. 2002). By contrast, Frias et al. (2012) found that males and females of the Magellanic penguin (*Spheniscus magellanicus*) from the Atlantic coast of Patagonia had similar median Hg levels in all age classes, although in adult males the range was greater than in adult females. The Hg levels that we found in the feathers of African penguins in this study did not differ significantly ($p > 0.05$) between males and females (Table 1) and were in the range 1.30 to 2.80 mg Kg^{-1} d.w. Our results support the hypothesis that sex-related variation in metal content does not occur in closely related penguins of the genus *Spheniscus*. Differences in diet of wild penguins may explain the variation in Hg concentrations between similar aged seabirds from different environments, while differing Hg concentrations between the sexes are thought to be a result of metabolic differences between the sexes (Frias et al. 2012). Some authors have suggested that male penguins could have more Hg than females because egg-laying may allow excretion of Hg (Becker et al. 2002; Falkowska 2013). Penguins have a unique molting pattern among birds; they renew all their feathers simultaneously, just before or just after the breeding period, and Hg concentrations in feathers represent Hg exposure in the period of time elapsed since the last molt. In fact, Hg excretion through the quill is a detoxification mechanism during premolting (Becker et al. 2002), and once the boom ends its growth, the blood transport channel atrophies leaving a permanent record until the next molt (Burger and Gochfeld 2002). There were no significant differences in Hg content between males and female in our study, although gender only seems to be of interpretive concern for species exhibiting different dietary habits between sexes (Robinson et al. 2012), and diet is very homogeneous in captive penguins.

3.2 Age-related differences

The concentrations of metals in feathers reflect the body burden at the time that the feathers are grown (Furness et al. 1986). Unlike most other avian orders, penguins have a unique molt in the year, with fasting periods of 2-3 weeks on average (Adams and Brow 1990).

Age is an important factor to be considered in exposure to chemicals in wildlife. Young animals could have higher exposure because they are often restricted to nesting sites or brooding sites, while adults tend to roam far from the source of contamination (Lodenius and Solonen 2013).

Conversely, in our study, juveniles and adults had the same patterns of exposure and we did not find age-related differences in the feathers of African penguins, except for selenium ($t = 2.42$, $df = 44$, $p < 0.05$; Figure 1b). Se is a metalloid trace element that birds and other wildlife need in small amounts to maintain good health (Ohlendorf and Heinz 2009). Se levels of 3.8 to 26 mg kg^{-1} d.w. (depending upon the species) in feathers results in mortality (Burger 1993), and 1.8 mg kg^{-1} d.w. results in sub-lethal adverse effects (Ohlendorf and Heinz 2009). Se levels in our samples (Table 1) ranged from 2.142 (juvenile females) to 2.656 mg kg $^{-1}$ (adults males), and were similar or higher than those detected by Metcheva et al. (2006) in the feathers of the Gentoo Penguin, but lower than those reported by Jerez et al. (2011) in the Adélie penguin (*Pygoscelis adeliae*) and in the Chinstrap penguin (*Pygoscelis Antarctica*). The increase in Se levels with age could be due to a chronic exposure to trace elements such as Cd and Hg, since Se is also known to have a detoxifying effect on these metals. Moreover, Ralston and Raymond (2010) suggested that the selenium:mercury molar ratio is an important consideration for understanding the toxic effects of mercury. While there is consensus that an excess of mercury compared to selenium is potentially hazardous, there is no consensus about the levels of selenium deemed necessary to reduce mercury toxicity. Different authors have suggested that Se:Hg molar ratios above 1 are probably protective (Ralston et al 2008; Peterson et al. 2009). While this molar ratio has been examined in fish because of the potential protective effects to humans consuming fish, recently Burger et al. (2014) suggested that it should be protective of brain function in birds and other animals as well. In fact, the Se:Hg molar ratio has been shown to vary significantly between tissues, with the highest ratios in brain and liver and the lowest in feathers (Burger et al. 2014). In the present study, the Se:Hg molar ratio was above 1, ranging from 2.51 (adult females) to 2.77 (adult males) and presumably protective for both genders (Table 2).

4. Conclusions

Sex-related effects should only be examined when males and females have been directly compared under similar conditions. This unique type of experimental approach can be successfully carried out with animals living in controlled environments, where the entire colony is fed with the same identical food supply. Moreover, feathers are ideal for monitoring exposure and inferring effects because they can be sampled non-invasively with minimal stress to the birds, especially relevant to endangered species. In particular, birds with a synchronous molt are good candidates for bioindicators of metal contamination. Investigating sex- and age-related variations in environmental contaminant concentrations in seabirds has produced contrasting results. In most studies on feathers, no age-related variations were detected for several trace elements, while gender-related variations were detected in species that exhibit different dietary habits between the sexes.

In the captive African penguins examined, Hg content in feathers seemed to reflect the bio-magnification phenomena through the penguin's fish diet but did not show any significant relationship with sex and age. However, arsenic seems to be gender-related in juveniles, probably due to a different metabolic rate at that phase of growth. Finally, selenium concentration was shown to increase with increasing age, according to its function of contrasting the toxicity of certain metals. The concentrations of the other 13 trace elements considered appeared to be unaffected by sex or age, but the relationships between concentrations of many trace elements in internal tissues and feathers concentrations remains to be determined.

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