Metals in feathers of African penguins (*Spheniscus demersus*): considerations for the welfare and management of seabirds under human care

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Abstract
Bird feathers have been proven to be reliable indicators of metal exposure originating from contaminated food and polluted environments. The concentrations of 15 essential and non-essential metals were investigated in African penguins (*Spheniscus demersus*) feathers from a Northwestern Italian zoological facility. These birds are exclusively fed with herring from the northeast Atlantic Ocean. Certain elements, such as Hg and Cd, reflected the bioaccumulation phenomena that occur through the marine food chain. The levels of Cr, Mn, and Ni were comparable to those registered in feathers of birds living in polluted areas. These results are important for comparative studies regarding the health, nutrition and welfare of endangered seabirds kept under human care.
Keywords: metal accumulation, biomonitoring, penguins, feathers.

Inorganic contaminants such as metals are major pollutants, which are persistent and ubiquitous in ecosystems due to their natural and anthropogenic origins (Abbasi et al. 2015). Essential trace elements include chromium (Cr), copper (Cu), cobalt (Co), iron (Fe), manganese (Mn), nickel (Ni), selenium (Se), tin (Sn), vanadium (V), and zinc (Zn). These elements are necessary for life but when they exceed physiological concentrations in tissues and organs they can be toxic (Barton & Schmitz, 2009). Non-essential trace elements include arsenic (As), cadmium (Cd), mercury (Hg), and lead (Pb), that can be tolerated by biota at very low levels, but become harmful upon bioaccumulation (Eisler 1981; Burger et al. 2008).
In recent years, feathers have become the method of choice to evaluate trace elements contamination in birds (Jerez et al. 2011; Carravieri et al. 2013; Abbasi et al. 2015; Abdullah et al. 2015). Indeed, in feathers, metals are bound to keratin, a sulfur-containing protein (Dauwe et al. 2000; Metcheva et al. 2006); and several metals have a strong affinity to keratin (Dmowski 1999). During growth, feathers are perfused from blood vessels, and metals ingested with food were incorporated into feather keratin structures. Then, metals concentrations in feathers can indicate the physiological condition of the bird during the time of active feather growth (Burger 1993).
Penguins (Order: *Sphenisciformes*; Family: *Spheniscidae*) are seabirds at the top of many marine food chains. Accordingly, penguins bioconcentrate metals in biologically available forms at several orders of magnitude above environmental levels (Markowski et al. 2013). The genus *Spheniscus* comprises four different extant species, which inhabit temperate and equatorial areas of the Southern Hemisphere (Schreiber & Burger 2002) and share common morphological traits and behavioral ecology (Williams 1995; Favaro et al. 2016). The African or Jackass Penguin (*Spheniscus demersus*) is a non-migratory seabird endemic to South Africa and Namibia, and it is the only penguin species that breeds in the African continent. African penguin juveniles undergo their first molt in spring/summer, between the ages of 12 and 23 months (Kemper et al. 2008). Adult penguins molt once a year, with a feather-shedding phase of 12.7±1.4 days (Randall et al. 1986). Accordingly, the discarded plumage allows investigation of the metals, which have been accumulated by the penguins since the previous molt.
In the wild, the African penguin feeds on pelagic schooling fish; prey size varies according to geographical location (Davis & Darby 1990). The current conservation status for the African penguin is "endangered", according to the Red List of Threatened Species of the International Union for Conservation of Nature (BirdLife International, 2013). Wild African penguin populations

have dramatically decreased, due to loss of habitat, reduced fish stocks and environmental pollution (Crawford et al. 2011). Consequently, in-situ conservation programs are becoming crucial. Moreover, African penguins are also included in many ex-situ conservation programs and are frequently kept and bred in zoos and aquaria worldwide African penguins are currently living in captivity (Blay & Côté, 2001). Seabirds in zoos and aquaria are often subject to a variety of dietary limitations. In particular, African penguins under human care are usually provided with food that is not fully representative of natural prey resources (Heat & Randall, 1985). European zoos mostly feed their birds with herring from the northeast Atlantic Ocean, i.e. wild-caught prey, which could have elevated levels of contaminants (Pohl & Hennings, 2009). Furthermore, penguins kept in captivity could be more directly exposed to anthropogenic contaminants than wild populations, due to the location of many zoos close to or within metropolitan areas. Metals have been shown to be related to variation in the plumage density (Eeva et al. 1998), reduction of genetic diversity (Eeva et al. 2006), low fledging success (Evers et al. 2008), decreased bone mineralization degree (Gangoso et al. 2009), altered humoral immune responsiveness (Snoeijs et al. 2004), aberrant incubation behavior, lethargy and asymmetric wing area (Evers et al. 2008).
Accordingly, our main aims were:
i) to assess Al, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Sn, Zn, and V concentrations in the feathers of a large captive colony of *Spheniscus demersus* in Italy;
ii) to increase the data available on penguin welfare in zoos, by evaluating their exposure to potentially toxic concentrations of essential and non-essential elements accumulated through food consumption.
We predict that captive penguins will bioaccumulate metal concentrations above those in their provided diet.

Materials and methods

Samples collection

Feathers were collected before the beginning of the molting season in 2014 from 49 African penguins housed at the Zoom Biopark, Torino (44°56' N, 7°25’ E). In this zoological facility, penguins were kept in an outdoor communal exhibit of 1500 m^2, which included a pond of 120 m^2 (maximum depth: 3 m). Feather samples were collected as previously described (Squadrone et al., 2016). In addition, the whole bodies of several herring (*Clupea harengus*) were collected in the same period from the penguins’ food stock. Fish were selected randomly, pooled, stored and then maintained at –20 °C prior to analysis. All fish were from a northeast Atlantic Ocean (FAO fishing area 27).

Analytical methods

Surface lipids and contaminants were removed from feathers following a protocol already described (Squadrone et al., 2016), then minced with a stainless steel scissors. Mercury was quantified with a Direct Mercury Analyzer (Milestone, Shelton, CT, USA) and the other elements by Inductively Coupled Plasma-Mass Spectrometry (Thermo Scientific, Bremen, Germany) after being subjected to microwave digestion as already described (Squadrone et al., 2016). 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.

Statistical analysis

Data were tested for normality by using the Kolmogorov-Smirnov test. As data distribution was non-normal and could not be satisfactorily transformed, a non-parametric Spearman’s rho was used to test for correlations between metal concentrations in penguin feathers. All analyses were performed in the SPSS version 20.0 for Macintosh. Alpha values were two-tailed and set at 0.05.

Results and discussion

The concentrations of metals in the feathers of the African penguins and in their food (herring) are shown in Table 1, and were in the decreasing order: Fe>Zn>Ni>Al>Cu>Cr>Mn>Se>Hg>Sn>Pb>Cd>V>Co>As. In Figure 1, bioaccumulation of certain metals in penguin's feathers in comparison to penguins' food are shown, while correlations between metals are represented in Figure 2.

Mercury and arsenic

Mercury is a contaminant of great interest in marine ecosystems. This toxic element bioaccumulates and biomagnifies in the marine food web, essentially through dietary uptake (Frias et al. 2012). Effects of mercury on birds include behavioral and neurodevelopmental deficits, impaired reproduction, and even lethality: sensitive birds can experience adverse effects at dietary concentrations of 0.05 to 0.50 mg kg^{-1} (Eisler, 1987). Seabirds are able to detoxify mercury and are therefore more resistant to its harmful effects (Ribeiro et al. 2009). Accordingly, the Hg levels we measured in penguin feathers were below the level related to harmful effects and comparable to levels reported by Falkowska et al. (2013a,b), in a colony of African penguins living in a Polish Zoo, which received an equivalent amount of herring from the Baltic Sea. The content of metals that we detected in this fish was similar to values in herring from the same Atlantic area reported by other authors (Polak-Juszczak 2009). As mercury is known to bioaccumulate through food chains (Lodenious & Solonen, 2013), it can be suggested that the presence of Hg in the feathers of this captive colony is due to the consumption of pelagic fish and the subsequent bioaccumulation in feathers (Figure 2).

Arsenic is assimilate by fish by ingesting particulate material suspended in water and by food ingestion (Višnjić-Jeftić et al. 2010). Arsenic concentration was found to decrease as the trophic level increases in food chains (Rahman et al. 2012). Accordingly, a higher As content in herring was detected compared to penguin feathers (Table 1). Currently, there are no previous reports concerning As contamination in captive seabirds, but the values found here are similar to those reported by Jerez and coauthors (2011) in feathers of wild Antarctic penguins.

Cadmium and lead

Cadmium is a very toxic element for biota and may cause reduction in growth rates and lethal effects at lower concentrations than other harmful elements such as mercury (Spahn & Sherry 1999). According to the literature, Cd levels in seabird feathers are usually less than 0.20 mg kg^{-1} d.w (Burger & Gochfeld, 2000). Burger also reported that Cd in feathers may cause adverse and toxic effects when exceeding levels of 0.1 to 2 mg kg^{-1}, and this effect is species dependent. In this study, there was an average Cd level close to the limit considered to be toxic in the African penguin feathers (Table 1). The Cd levels found here deserve further investigation, considering that in herrings Cd concentration was close to the instrumental LOQ (Figure 2). However, we suggest that cadmium is subject to bioaccumulation following dietary intake during penguin's lifetime. In fact, it is well known that cadmium disturbs calcium homeostasis, due to the ability of Cd to mimic Ca during bone ossification and development. Thus, Cd concentration in feathers reflects the mobilization from internal tissues and may represent a biomarker of greater whole body exposure and bioaccumulation.

Lead is a neurotoxin that causes a decrease in growth, learning ability, and metabolism (Burger & Gochfeld, 2000). Eisler (1988) suggested that average Pb levels of 50 mg kg^{-1} d.w. in the diet may produce adverse effects in avian predators, but levels as low as 0.10 mg kg^{-1} d.w. have been correlated with learning deficits in sensitive vertebrates. Pb is not metabolically regulated and can accumulate in bird feathers at high concentrations; therefore, it is one of the most suitable metals for monitoring anthropogenic pollution using birds (Metcheva et al. 2011). Due to the high affinity of Pb for sulfur, lead is excreted in feathers, presumably bound to the sulfhydryl groups in keratin (Sterner, 2010). Harmful effects in birds were observed at levels of 4 mg kg^{-1} d.w. in feathers (Eisler, 1988), although seabirds can often tolerate higher concentrations. Overall, the Pb levels measured here (Table 1) were below the level of concern, and in the penguins' food, the Pb content was negligible.

Aluminum and tin

Little is known about the toxicity of Al in birds, although high levels have been associated with impaired breeding, reduction in clutch size, defective eggshell formation, and intrauterine bleeding (Nyholm, 1981). Al concentrations above 1000 mg kg^{-1} in food could be toxic for young birds (Sparling et al. 1997). Al is likely to have a high affinity with feathers because several seabirds exhibited the highest levels in this integumentary structure (Lucia et al. 2010). The mean concentration found in this study was two orders of magnitude higher than in the penguins' food (Table 1). This could be the result of an accumulation phenomenon following dietary intake.

Tin and its compounds are generally thought relatively immobile in food chains and data are not still available for tin in captive birds. Values reported here are comparable to those obtained by Burger and Gochfeld (2000) in seabirds from the northern Pacific Ocean.

Iron and manganese

Iron is an essential element for biota, but could become toxic in high doses (Thomas & McGill, 2008). Fe originates naturally from rock and soil, but anthropogenic activities also contribute to its release in the environment (Abdullah et al. 2015). Iron was the most abundant element detected in penguin feathers in this study. These results also indicate a high availability of this metal in the penguins' food. Therefore, further investigations in other organs are needed to evaluate possible Fe bioaccumulation in captive penguins, and its possible toxic effects at high concentrations.

Manganese concentrations (Table 1) were detected at one order of magnitude higher than those reported in feathers of wild seabirds (Ribeiro et al. 2009), but were similar to those found in birds living in highly contaminated areas (Abdullah et al. 2015). This trace element enters the food chain, in fact, an elevated level was detected in penguin's food (Table 1, Figure 2), resulting in bioaccumulation in feathers.

Copper and zinc

According to the literature, copper and zinc do not bioaccumulate through food chains, but are regulated by organisms (Adriano, 2001). The copper levels detected in the feathers of the African penguins studied here were similar to those detected in the feathers of wild seabirds from other parts of the world, such as the Southwest Atlantic Coast of France and Antartica (Barbieri et al. 2010, Lucia et al. 2010). Moreover, the Cu level measured in herring was in the range reported by other authors in fish from the same area, and was of no toxicological concern.

Zinc is an essential element in the formation of feathers, and birds have been reported to accumulate large amounts of this element (Deng et al. 2007). Zn levels measured here were in accordance with the high concentration ranges reported in various bird species around the world. It was suggested that high Zn levels could be related to an adaptive process of the African penguins to mercury and cadmium contamination, as an increase in Zn levels is known to reduce the toxic effect of these heavy metals (Jerez et al. 2011).

Chromium and nickel

Chromium was detected in all samples, reflecting its role as an essential element. However, neurotoxic effects in birds were already suggested and results reported here are within the upper range of Cr concentrations found in bird feathers (Burger, 1993). In particular, the feathers of the African Penguins examined showed Cr levels of one order of magnitude higher than those detected in the feathers of wild seabirds (Burger & Gochfeld, 2009, 2010).

Nickel is essential for animal nutrition, but data on Ni levels in seabirds are still scarce. It has been suggested that the tissues of wild birds from uncontaminated environments should contain between 0.10 and 5.0 mg kg^{-1} d.w. (Outridge & Scheuhammer, 1993), but scarce information is available on the toxicity of Ni in birds. However, adverse effects such as genotoxicity and immunotoxicity were suggested for this metal (Das, 2008). The Ni levels measured here in penguin feathers are comparable with those obtained by Abdullah and coauthors (2015) in birds living in an industrial area in Pakistan. Anthropogenic sources like mining and waste incineration are known increase Ni environmental levels (ATSDR, 2005). Comparison with Cr and Ni levels in captive seabirds was not possible due to the scarcity of data regarding these trace elements, but we found that they were particularly bio accumulated in penguin's feathers.

Selenium, cobalt and vanadium

Selenium is a metalloid that birds and other wildlife require in small amounts for biological functions (Ohlendorf & Heinz, 2009). However, at high concentrations, selenium can be very toxic and subject to homeostatic regulation. In feathers, levels of 3.8 to 26 mg kg^{-1} (according to species) result in severe adverse effects, such as mortality of eggs; moreover, Heinz (1996) reported that concentrations of 1.8 mg kg^{-1} could result in sublethal adverse effects in birds. Selenium levels in the feathers of the African penguins that were analyzed in this study were below the values reported to be toxic, and the herring content did not pose any risk for the penguins.

Cobalt is a relatively rare element of the earth's crust, essential to mammals in the form of cobalamin (vitamin B_{12}). Co is a naturally occurring element found in rocks, soil, water, plants, and animals, and has diverse industrial importance. Vanadium has variable concentrations in biota, due to different dietary and background levels. Co and V levels in penguin feathers were relatively low and were below the LOQ in the penguins' food.

There were a number of positive significant relationships between concentrations of metals in bird feathers, suggesting common uptake and storage pathways, or similar regulation and detoxification processes. Specifically, in African Penguin feathers, we found three different positive correlations between pairs of elements, suggesting that penguin feathers accumulate these metals during growth, due to the existence of a high blood flow. This accumulation in feathers allows the elimination of partial contents of toxic metals from the organism. In fact, we observed a positive correlation between Fe and Cr (Spearman's rho $\dot{\rho} = 0.835$, $N = 49$, $p < 0.001$), Cu and Ni (Spearman's rho $\dot{\rho} = 0.806$, $N = 49$, $p < 0.001$), Co and V (Spearman's rho $\dot{\rho} = 0.770$, $N = 49$, $p < 0.001$).

Conclusions

Zoos and aquaria worldwide aim to contribute to the *ex-situ* conservation of a variety of endangered seabird species, including penguins. In order to increase the reproductive success, decrease the incidence of pathologies, and avoid genotoxic effects, it is essential to monitor and minimize the level of exposure to essential and non-essential heavy metals in seabirds maintained under human care. According to the literature, there is usually a link between metal levels in the diet of birds and levels detected in their feathers. The captive colony of African penguins studied here received a specific and homogeneous diet (herring from the northeast Atlantic Ocean) which revealed the effect of food on the degree of exposure to essential and non-essential metals. For this reason, it can be recommended that captive colonies of penguins and seabirds, in general, should be fed with a varied diet, where possible, which is representative of their natural diet, avoiding the use of only one pelagic fish species.

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Ethical statement - This research conformed to the Ethical Guidelines for the Conduct of Research on Animals by Zoos and Aquariums (WAZA, 2005) , and was carried out with the approval of the Ethical Committee of the Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta (11168; 14 July 2014).

Table 1. Metals concentrations (mg kg^{-1} d.w., mean ± SD) in feathers and in food of the examined African Penguin specimens (n=49).

Element	Feathers	Herring
Al	38 ±16	0.27±0.05
As	0.15±0.06	2.0±0.04
Cd	0.33±0.2	<0.010
Co	0.16±0.1	<0.010
Cr	22±77	0.04±0.005
Cu	23 ±12	1.0±0.01
Fe	183±543	9.7±0.14
Hg	2.2±0.59	0.041±0.01
Mn	15±17	0.32±0.10
Ni	58±45	0.05±0.003
Pb	0.56±0.38	0.02±0.003
Se	2.4±0.52	0.55±0.01
Sn	1.2±7.3	0.02±0.003
V	0.28±0.22	<0.010
Zn	98±32	4.4±0.40

Figure 1. Metals bioaccumulation in penguin's feathers from food.

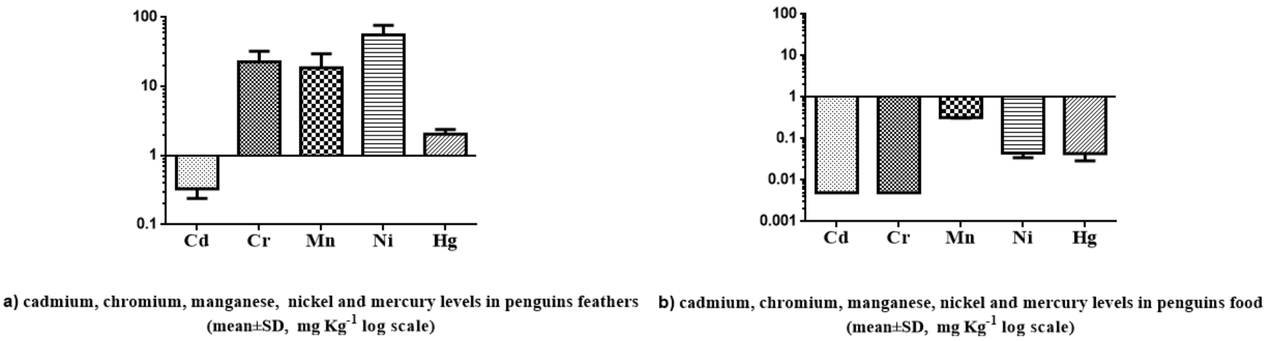
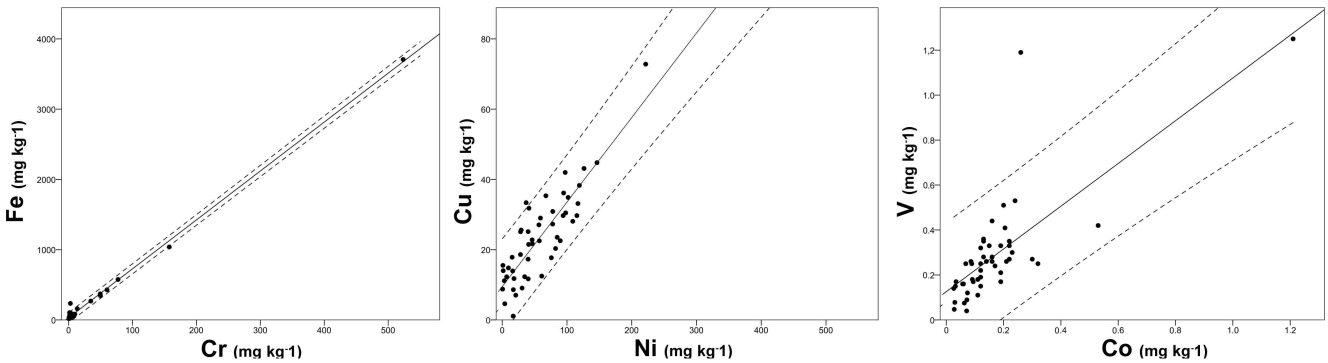


Figure 2. Trace elements whose bioaccumulation was found to be correlated in penguin feathers. Dotted lines represent the 95% Confidence Interval.



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