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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1660007> since 2022-12-21T11:26:42Z

Published version:

DOI:10.1007/s00128-018-2293-9

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

3
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10
11 **Abstract**

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

20 **Keywords:** metal accumulation, biomonitoring, penguins, feathers.

21
22 Inorganic contaminants such as metals are major pollutants, which are persistent and ubiquitous in
23 ecosystems due to their natural and anthropogenic origins (Abbasi et al. 2015). Essential trace
24 elements include chromium (Cr), copper (Cu), cobalt (Co), iron (Fe), manganese (Mn), nickel (Ni),
25 selenium (Se), tin (Sn), vanadium (V), and zinc (Zn). These elements are necessary for life but
26 when they exceed physiological concentrations in tissues and organs they can be toxic (Barton &
27 Schmitz, 2009). Non-essential trace elements include arsenic (As), cadmium (Cd), mercury (Hg),
28 and lead (Pb), that can be tolerated by biota at very low levels, but become harmful upon
29 bioaccumulation (Eisler 1981; Burger et al. 2008).

30 In recent years, feathers have become the method of choice to evaluate trace elements
31 contamination in birds (Jerez et al. 2011; Carravieri et al. 2013; Abbasi et al. 2015; Abdullah et al.
32 2015). Indeed, in feathers, metals are bound to keratin, a sulfur-containing protein (Dauwe et al.
33 2000; Metcheva et al. 2006); and several metals have a strong affinity to keratin (Dmowski 1999).
34 During growth, feathers are perfused from blood vessels, and metals ingested with food were
35 incorporated into feather keratin structures. Then, metals concentrations in feathers can indicate the
36 physiological condition of the bird during the time of active feather growth (Burger 1993).

37 Penguins (Order: *Sphenisciformes*; Family: *Spheniscidae*) are seabirds at the top of many marine
38 food chains. Accordingly, penguins bioconcentrate metals in biologically available forms at several
39 orders of magnitude above environmental levels (Markowski et al. 2013). The genus *Spheniscus*
40 comprises four different extant species, which inhabit temperate and equatorial areas of the
41 Southern Hemisphere (Schreiber & Burger 2002) and share common morphological traits and
42 behavioral ecology (Williams 1995; Favaro et al. 2016). The African or Jackass Penguin
43 (*Spheniscus demersus*) is a non-migratory seabird endemic to South Africa and Namibia, and it is
44 the only penguin species that breeds in the African continent. African penguin juveniles undergo
45 their first molt in spring/summer, between the ages of 12 and 23 months (Kemper et al. 2008). Adult
46 penguins molt once a year, with a feather-shedding phase of 12.7±1.4 days (Randall et al. 1986).
47 Accordingly, the discarded plumage allows investigation of the metals, which have been
48 accumulated by the penguins since the previous molt.

49 In the wild, the African penguin feeds on pelagic schooling fish; prey size varies according to
50 geographical location (Davis & Darby 1990). The current conservation status for the African
51 penguin is “endangered”, according to the Red List of Threatened Species of the International
52 Union for Conservation of Nature (BirdLife International, 2013). Wild African penguin populations

53 have dramatically decreased, due to loss of habitat, reduced fish stocks and environmental pollution
54 (Crawford et al. 2011). Consequently, in-situ conservation programs are becoming crucial.
55 Moreover, African penguins are also included in many ex-situ conservation programs and are
56 frequently kept and bred in zoos and aquaria worldwide African penguins are currently living in
57 captivity (Blay & Côté, 2001). Seabirds in zoos and aquaria are often subject to a variety of dietary
58 limitations. In particular, African penguins under human care are usually provided with food that is
59 not fully representative of natural prey resources (Heat & Randall, 1985). European zoos mostly
60 feed their birds with herring from the northeast Atlantic Ocean, i.e. wild-caught prey, which could
61 have elevated levels of contaminants (Pohl & Hennings, 2009). Furthermore, penguins kept in
62 captivity could be more directly exposed to anthropogenic contaminants than wild populations, due
63 to the location of many zoos close to or within metropolitan areas. Metals have been shown to be
64 related to variation in the plumage density (Eeva et al. 1998), reduction of genetic diversity (Eeva et
65 al. 2006), low fledging success (Evers et al. 2008), decreased bone mineralization degree (Gangoso
66 et al. 2009), altered humoral immune responsiveness (Snoeijs et al. 2004), aberrant incubation
67 behavior, lethargy and asymmetric wing area (Evers et al. 2008).

68 Accordingly, our main aims were:

- 69 i) to assess Al, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, Sn, Zn, and V concentrations in the
70 feathers of a large captive colony of *Spheniscus demersus* in Italy;
- 71 ii) to increase the data available on penguin welfare in zoos, by evaluating their exposure to
72 potentially toxic concentrations of essential and non-essential elements accumulated through food
73 consumption.

74 We predict that captive penguins will bioaccumulate metal concentrations above those in their
75 provided diet.

76 **Materials and methods**

77 *Samples collection*

78 Feathers were collected before the beginning of the molting season in 2014 from 49 African
79 penguins housed at the Zoom Biopark, Torino (44°56' N, 7°25' E). In this zoological facility,
80 penguins were kept in an outdoor communal exhibit of 1500 m², which included a pond of 120 m²
81 (maximum depth: 3 m). Feather samples were collected as previously described (Squadrone et al.,
82 2016). In addition, the whole bodies of several herring (*Clupea harengus*) were collected in the
83 same period from the penguins' food stock. Fish were selected randomly, pooled, stored and then
84 maintained at -20 °C prior to analysis. All fish were from a northeast Atlantic Ocean (FAO fishing
85 area 27).

86 *Analytical methods*

87 Surface lipids and contaminants were removed from feathers following a protocol already described
88 (Squadrone et al., 2016), then minced with a stainless steel scissors. Mercury was quantified with a
89 Direct Mercury Analyzer (Milestone, Shelton, CT, USA) and the other elements by Inductively
90 Coupled Plasma-Mass Spectrometry (Thermo Scientific, Bremen, Germany) after being subjected
91 to microwave digestion as already described (Squadrone et al., 2016). Multi-elemental
92 determination was performed with ICP-MS after daily optimization of instrumental parameters and
93 using an external standard calibration curve; rodium and germanium were used as internal
94 standards. Analytical performances were verified by processing Certified Reference Materials
95 (Dogfish liver -DOLT-4 from the National Research Council of Canada, and Oyster Tissue-SRM
96 1566b from the National Institute of Standard and Technology), along with blank reagents in each
97 analytical session. The recoveries for reference materials ranged from 85 to 120% for DOLT-4 and
98 from 82 to 117% for SRM 1566b. The limit of quantitation (LOQ) was 0.010 mg Kg⁻¹ for all
99 elements.

100 *Statistical analysis*

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

105 **Results and discussion**

106 The concentrations of metals in the feathers of the African penguins and in their food (herring) are
107 shown in Table 1, and were in the decreasing order:

108 Fe>Zn>Ni>Al>Cu>Cr>Mn>Se>Hg>Sn>Pb>Cd>V>Co>As. In Figure 1, bioaccumulation of
109 certain metals in penguin's feathers in comparison to penguins' food are shown, while correlations
110 between metals are represented in Figure 2.

111 *Mercury and arsenic*

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

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

132 *Cadmium and lead*

133 Cadmium is a very toxic element for biota and may cause reduction in growth rates and lethal
134 effects at lower concentrations than other harmful elements such as mercury (Spahn & Sherry
135 1999). According to the literature, Cd levels in seabird feathers are usually less than 0.20 mg kg⁻¹
136 d.w (Burger & Gochfeld, 2000). Burger also reported that Cd in feathers may cause adverse and
137 toxic effects when exceeding levels of 0.1 to 2 mg kg⁻¹, and this effect is species dependent. In this
138 study, there was an average Cd level close to the limit considered to be toxic in the African penguin
139 feathers (Table 1). The Cd levels found here deserve further investigation, considering that in
140 herrings Cd concentration was close to the instrumental LOQ (Figure 2). However, we suggest that
141 cadmium is subject to bioaccumulation following dietary intake during penguin's lifetime. In fact, it
142 is well known that cadmium disturbs calcium homeostasis, due to the ability of Cd to mimic Ca
143 during bone ossification and development. Thus, Cd concentration in feathers reflects the
144 mobilization from internal tissues and may represent a biomarker of greater whole body exposure
145 and bioaccumulation.

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

157 *Aluminum and tin*

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

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

168 *Iron and manganese*

169 Iron is an essential element for biota, but could become toxic in high doses (Thomas & McGill,
170 2008). Fe originates naturally from rock and soil, but anthropogenic activities also contribute to its
171 release in the environment (Abdullah et al. 2015). Iron was the most abundant element detected in
172 penguin feathers in this study. These results also indicate a high availability of this metal in the
173 penguins' food. Therefore, further investigations in other organs are needed to evaluate possible Fe
174 bioaccumulation in captive penguins, and its possible toxic effects at high concentrations.

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

180 *Copper and zinc*

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

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

193 *Chromium and nickel*

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

199 Nickel is essential for animal nutrition, but data on Ni levels in seabirds are still scarce. It has been
200 suggested that the tissues of wild birds from uncontaminated environments should contain between
201 0.10 and 5.0 mg kg⁻¹ d.w. (Outridge & Scheuhammer, 1993), but scarce information is available on
202 the toxicity of Ni in birds. However, adverse effects such as genotoxicity and immunotoxicity were
203 suggested for this metal (Das, 2008). The Ni levels measured here in penguin feathers are
204 comparable with those obtained by Abdullah and coauthors (2015) in birds living in an industrial
205 area in Pakistan. Anthropogenic sources like mining and waste incineration are known increase Ni
206 environmental levels (ATSDR, 2005). Comparison with Cr and Ni levels in captive seabirds was
207 not possible due to the scarcity of data regarding these trace elements, but we found that they were
208 particularly bio accumulated in penguin's feathers.

209 *Selenium, cobalt and vanadium*

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

217 Cobalt is a relatively rare element of the earth's crust, essential to mammals in the form of
218 cobalamin (vitamin B₁₂). Co is a naturally occurring element found in rocks, soil, water, plants, and
219 animals, and has diverse industrial importance. Vanadium has variable concentrations in biota, due
220 to different dietary and background levels. Co and V levels in penguin feathers were relatively low
221 and were below the LOQ in the penguins' food.

222 There were a number of positive significant relationships between concentrations of metals in bird
223 feathers, suggesting common uptake and storage pathways, or similar regulation and detoxification
224 processes. Specifically, in African Penguin feathers, we found three different positive correlations
225 between pairs of elements, suggesting that penguin feathers accumulate these metals during growth,
226 due to the existence of a high blood flow. This accumulation in feathers allows the elimination of
227 partial contents of toxic metals from the organism. In fact, we observed a positive correlation
228 between Fe and Cr (Spearman's rho $\hat{\rho}$ = 0.835, N = 49, p < 0.001), Cu and Ni (Spearman's rho $\hat{\rho}$ =
229 0.806, N = 49, p < 0.001), Co and V (Spearman's rho $\hat{\rho}$ = 0.770, N = 49, p < 0.001).

230 **Conclusions**

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

242
243 The authors would like to thank Zoom Torino S.p.A. (www.zoomtorino.it), and in particular Dr.
244 Daniel Sanchez, Dr. Valentina Isaja, Dr. Laura Ozella, and Dr. Sara Piga for their help during
245 collection of samples. Kim Maciej is acknowledged for providing holding data for the genus
246 *Spheniscus*. Livio Favaro was supported during the writing of this manuscript by the University of
247 Torino through a MIUR co-financed postdoctoral fellowship.

248 The authors also thank the editor and the anonymous reviewers for useful suggestions and
249 comments on an earlier version of this manuscript.

250 *Ethical statement* - This research conformed to the Ethical Guidelines for the Conduct of Research
251 on Animals by Zoos and Aquariums (WAZA, 2005), and was carried out with the approval of the
252 Ethical Committee of the Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta
253 (11168; 14 July 2014).

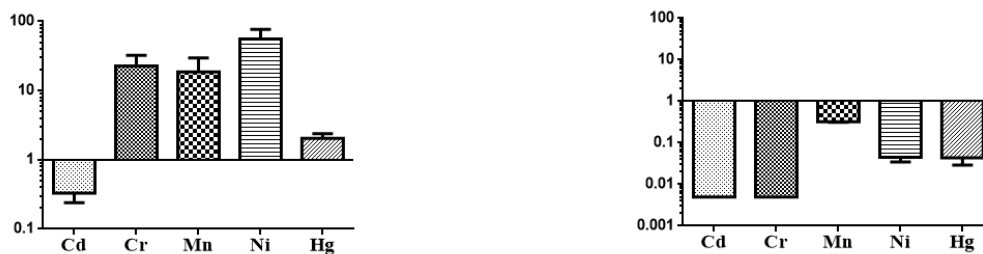
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Table 1. Metals concentrations (mg kg⁻¹ d.w., mean ± SD) in feathers and in food of the examined African Penguin specimens (n=49).

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

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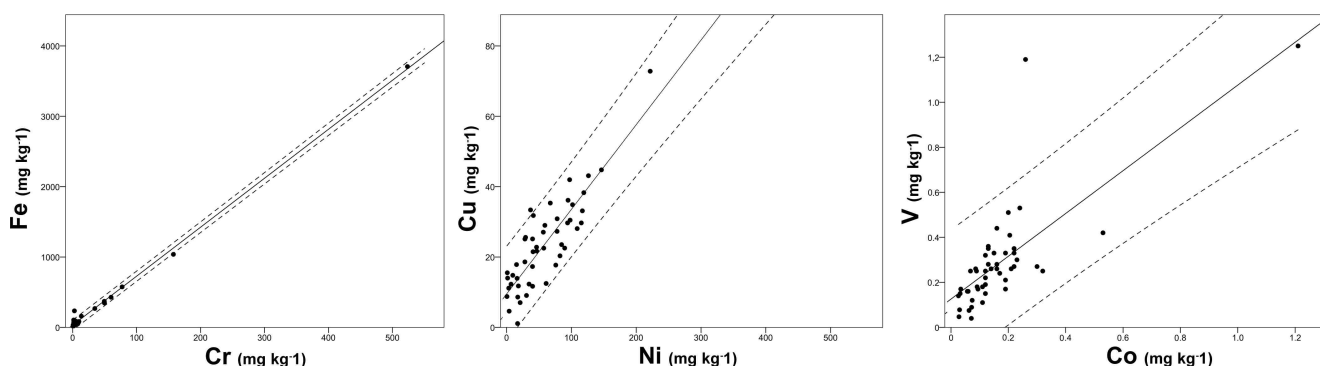
Figure 1. Metals bioaccumulation in penguin's feathers from food.



a) cadmium, chromium, manganese, nickel and mercury levels in penguins feathers (mean±SD, mg Kg⁻¹ log scale) b) cadmium, chromium, manganese, nickel and mercury levels in penguins food (mean±SD, mg Kg⁻¹ log scale)

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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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