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Humboldt penguins' feathers as bioindicators of metal exposure

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Abstract

Avian feathers have the potential to accumulate trace elements originating from contaminated food and polluted environments. In fact, in feathers, metals bind to keratin, a sulphur-containing protein for which several metals have a strong affinity. Here, the concentrations of 18 essential and non-essential elements were investigated in a Humboldt penguin (*Spheniscus humboldti*) colony housed at the Acquario di Cattolica (Italy). This species is listed as vulnerable in the *Red List* of the International Union for Conservation of Nature. According to the literature, there is usually a link between metal levels in the diet of birds and levels detected in their feathers. Thus, metals were also determined in the penguins' food (capelin, *Mallotus villosus*). We hypothesize that the controlled conditions in which birds are kept in captivity, and the homogeneous diet that they follow could allow a better understanding of metal bioaccumulation (such as mercury) or bio-dilution (such as arsenic) in the marine food chain, indicated by penguins' feathers.

Moreover, comparisons with our previous investigations performed on an *ex-situ* African penguin (*Spheniscus demersus*) colony suggest that penguins living indoors have lower body burden of metals than those living outdoors. Indeed, environmental contaminants usually found in areas subjected to anthropogenic impact, where zoos and aquaria are often located, are not accumulated to levels of concern.

Keywords: seabirds, trace elements, feathers, bioaccumulation.

1. Introduction

The Humboldt Penguin (*Spheniscus humboldti*) is one of the 18 existing species of penguin and has been classified in the Red List of endangered species as having a vulnerable status by the International Union for Conservation of Nature (IUCN) (BirdLife International. 2016).

Four species belong to this genus (*Spheniscus*), namely the Humboldt penguin, the Magellanic penguin (*Spheniscus magellanicus*), the African penguin (*Spheniscus demersus*) and the Galapagos penguin (*Spheniscus mendiculus*), which are found in South Africa and South America (Baker *et al.* 2006). The Humboldt penguin reproduces along the coasts and islands of Chile and Peru, from the region of Valparaíso to the Island Lobos de Tierra (Murphy 1936). The population is composed of several thousand specimens; Chile and Peru have implemented the Washington Convention on the International Trade of wild species of endangered fauna and flora (CITES) as a national law (Paredes *et al.* 2003), prohibiting hunting, holding, capturing, transporting and exporting for commercial purposes (Iriarte 1999).

Humboldt penguin colonies are also conserved and bred *ex-situ* in aquaria all over the world, including Italy. Penguins in zoos and aquaria are excellent model organisms to study metal bioaccumulation through food and, according to the literature, there is usually a link between metal levels in the diet of birds and levels detected in their feathers (Squadrone *et al.* 2018; Markowski *et al.* 2013; Falkowska *et al.* 2013 a, b).

For the two last decades, bird's feathers have, in fact, become one of the best choices to investigate metal pollution in natural habitats (Burger 1993; Dmowski 1993; Burger and Gochfeld 2000; 2009; Dauwe 2000; Deng *et al.* 2007; Burger *et al.* 2008; Lucia *et al.* 2010; Markowski *et al.* 2013), especially in penguins (Metcheva *et al.* 2006, 2011; Jerez *et al.*, 2011; Frias *et al.*, 2012; Carravieri *et al.* 2013; Lodenious and Solonen 2013; Squadrone *et al.* 2016, 2018).

Concentrations of metals in bird feathers reflect the physiological state during the time of active feather growth, while metal levels in blood only reflect short-time exposure to contaminants (Burg-

er 1993); moreover, feather collection has the advantage of being a non-invasive method of investigation.

Thus, analysing metal levels in penguin feathers is crucial for assessing the health and welfare of captive seabirds, which could be subjected to several dietary limitations in aquaria and zoos (Squadrone *et al.* 2018).

In their natural habitat, penguins are predominantly piscivorous, feeding on various species of fish, small crustaceans and squid. In captivity, they usually follow a very homogeneous diet mainly composed of a single fish species, as already described by previous investigations regarding captive colonies of *S. demersus* (Falkowska *et al.* 2013a,b; Squadrone *et al.* 2018).

We had the opportunity to study the metal content of feathers in Humboldt penguins at the Acquario di Cattolica (Rimini, Italy), which were exclusively fed with capelin from Norway. This is the first study aimed at investigating metal transfer of 18 trace elements from food to feathers in this species; moreover, investigations regarding metals in *S. humboldti* are very scarce. In fact, to our knowledge, only mercury levels have been investigated, by Álvarez-Varas *et al.* (2018) in Humboldt feathers from the Chilean and Antarctic coasts, while the concentrations of six metals (arsenic, cadmium, copper mercury, lead and zinc) have been investigated in Humboldt excreta from the northern coast of Chile (Celis *et al.* 2014).

This Italian Humboldt penguin colony represents a simplified marine food chain, with no interference from outdoor environments, and we aimed at testing the hypothesis that some metals, e.g. mercury, bio-magnify and are consequently present at higher levels in feathers than in fish, while others, such as arsenic, decrease as the trophic level increases in food chains. To verify this hypothesis, Humboldt penguins' feathers and food were analysed for aluminium (Al), antimony (Sb), arsenic (As), beryllium (Be), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), nickel (Ni), selenium (Se), tin (Sn), thallium (Tl), vanadium (V) and zinc (Zn).

Moreover, due to the scarcity of data regarding captive penguins, we were interested in comparing

the body burden of metals in this *S. humboldti* colony, with concentrations previously found in another species of the genus *Spheniscus*, i.e. African penguins from another Italian zoological facility (Squadrone *et al.* 2018). The second hypothesis that we tested was to determine if the food provided to the Humboldt penguin colony had a metal content comparable to that of the food provided to the previously studied African penguin colony, then it followed that metal levels in the penguins' feathers should also have comparable concentrations.

94

2. Materials and methods

2.1 Sample collection

The Humboldt penguin colony (Figure 1) was composed of 12 adult penguins (8 females and 4 males) which were housed in an indoor exhibit of the Acquario di Cattolica (Rimini, Italy), with a total area of 75 m², including a salt-water tank of 35 m² (with a maximum depth of 2 m). Feathers were collected from moulting penguins and at the same time, penguins' food samples, capelin (*Mallotus villosus* from Norway) were also collected. All samples were pooled and stored at –20°C for further laboratory analyses.

2.2 Analytical methods

Surface lipids and contaminants were removed from feathers as previously described (Squadrone *et al.* 2016; 2018). Feathers were then minced and subjected to microwave digestion utilizing an ultra-wave oven (ETHOS 1, Milestone,) with 7 mL of HNO₃ (70% v/v) and 1.5 mL of H₂O₂ (30% v/v). Mercury was quantified using a Direct Mercury Analyzer (Milestone, Shelton, CT, USA) and the other elements were measured by Inductively Coupled Plasma-Mass Spectrometry (Thermo Scientific, Bremen, Germany), following the protocols previously described (Squadrone *et al.* 2016, 2018). The limit of quantification (LOQ) for all elements was 0.010 mg kg⁻¹. The analytical methods were validated according to UNI CEI EN ISO/IEC 17025 (General Requirements for the Competence of Testing and Calibration Laboratories).

2.3 Statistical analysis

114 The unpaired two-sample t-test was used to compare metal levels in the feathers between the two
115 penguin species (*Spheniscus humboldti*, in this study, and *Spheniscus demersus*, previous
116 investigation) and in the two fish species (capelin and herring, respectively). A conservative alpha
117 level of 0.01 was used. The Graph Pad Statistics Software Version 6.0 (GraphPad Software, Inc.,
118 USA) was used for statistical evaluations.

119 Results were considered statistically significant at p values of < 0.01. Graph Pad Statistics Software
120 Version 6.0 (GraphPad Software, Inc., USA) was used for statistical evaluations.

121

122 3. Results

123 Trace elements (in mg kg⁻¹) were found in *S. humboldti* feathers with the following decreasing
124 mean concentrations (Table 1): Zn (50) > Fe (17) > Al (12) > Cu (11) > Hg (2.8) > Mn (2.6) > Se
125 (0.85) > Ni (0.78) > Cr (0.54) > Pb (0.18) > As (0.14) > V (0.076) > Cd (0.060) > Sn (0.031) > Co
126 (0.027); Be, Sb and Tl were < LOQ. In the penguins' food (capelin, Table 1) the trend was the
127 following: Fe (11) > Zn (7.2) > As (1.8) > Cu (0.85) > Mn 0.42) > Se (0.26) > Al (0.21) > Ni
128 (0.076) > Cd (0.054) > V (0.052) > Hg (0.022) > Cr (0.012) > Pb (0.010); Be, Co, Sb, Sn and Tl
129 were < LOQ.

130 In Figure 1, a graphical comparison between metal levels in herring (African penguin's food) and
131 capelin (Humboldt penguin food) is shown. Metal levels were comparable in these two fish species,
132 with the exception of Cd and V, which were higher in capelin than in herring, and Sn, which was
133 only detectable in herring. To facilitate the graphical representation, values < LOQ (0.010 mg Kg⁻¹)
134 were represented with half of the LOQ (0.005 mg Kg⁻¹).

135 In Figure 2, a graphical comparison between metal levels in the feathers of African and Humboldt
136 penguins is presented. Differences were statistical significant (p < 0.01) for all metals, with the
137 exception of mercury and arsenic, which showed very similar levels in the two species.

138

139 4. Discussion

140 4.1 *Comparison with the literature regarding wild penguins and consideration about the use of*
141 *seabirds as bio-monitors.*

142 Seabirds play an important role as indicators of environmental problems in aquatic
143 ecosystems and species, as they are at a high position in the food chain and are particularly
144 threatened by toxic chemicals, due to their persistence and bio-magnification capacity. Moreover,
145 the widespread distribution of seabird species facilitates comparisons between ecosystems in
146 different countries. Bio-monitoring of environmental chemicals in wild birds has been employed in
147 several countries since the 1960s (Becker 2003) to assess long-term temporal and spatial trends of
148 chemical contaminants and to estimate their rates of change. There is a huge amount of literature on
149 seabirds' feathers used as bio-indicators of trace elements in aquatic ecosystems, and there have
150 been several studies performed in the wild that utilized penguin feathers for environmental metal
151 bio-indication. Penguins, in fact, are at the top of the marine food chain and are suitable sentinels to
152 investigate the presence of metals in the environment. However, the majority of investigations have
153 only focused on mercury levels in feathers (e.g. Álvarez-Varas *et al.*, 2018; Becker *et al.*, 2016;
154 Brasso *et al.*, 2015; Carravieri *et al.*, 2013; Frias *et al.*, 2012).

155 Mercury is a pervasive contaminant with no biological function in living beings, and its presence in
156 the environment is due to anthropogenic and natural sources. After its deposition in water ecosys-
157 tems, marine bacteria convert inorganic Hg into the more toxic and bioavailable organic form,
158 methylmercury (MeHg), which bio-magnifies through the marine trophic web. Mercury can exert
159 harmful effects on birds, such as neurodevelopmental, immunological and endocrine deficits, as
160 well as impaired reproduction. However, seabirds are known to have a higher tolerance to Hg than
161 terrestrial birds (Ribeiro *et al.* 2009). Being more exposed to Hg in the marine environment, sea-
162 birds have evolved more efficient mechanisms of detoxification; feathers represent the sequestration
163 site of mercury during their growth (Burger *et al.* 2011), and mercury concentrations in feathers are
164 assumed to represent more than 90% of the body burden of mercury in birds (Bearhop *et al.* 2000).

165 To our knowledge, there are no data regarding levels of trace elements in feathers of the Humboldt
166 penguins in the wild or in captivity, apart from the recent study by Álvarez-Varas and co-authors
167 (2018) focusing on mercury. They found a Hg mean level of 2.4 mg Kg⁻¹ (range 2.2 – 2.7 mg Kg⁻¹)
168 in feathers of *S. humboldti* from the Chilean and Antarctic coasts, and did not find significant differ-
169 ences between sexes. In our Humboldt colony, the Hg mean value was 2.8 mg Kg⁻¹ (Table 1), and
170 we also did not find any gender-related differences.

171 Several investigations from different parts of the world have reported Hg levels in feathers from
172 different penguin species such as the Gentoo penguin, *Pygoscelis papua*, the Chinstrap penguin,
173 *Pygoscelis antarctica*, the Adelie penguin, *Pygoscelis adeliae* (Metcheva *et al.* 2006; Jerez *et al.*
174 2011; Brasso *et al.* 2015; Becker *et al.* 2016); the Little penguin, *Eudyptula minor* (Dunlop *et al.*
175 2013; Brasso *et al.* 2015; Finger *et al.* 2015); the Rockhopper penguin, *Eudyptes chrysocome*
176 (Carravieri *et al.* 2013; Brasso *et al.* 2015); the African penguin, *Spheniscus demersus*, the
177 Emperor penguin, *Aptenodytes forsteri* (Brasso *et al.* 2015); the King penguin, *Aptenodytes*
178 *patagonicus* and the Macaroni penguin, *Eudyptes chrysolophus* (Carravieri *et al.* 2013).

179 All these studies have reported different mercury levels in penguins' feathers, mostly related to the
180 degree of pollution in the environment in which these species live; however, mercury content was
181 found to be strictly related with foraging, and generally piscivorous species have higher Hg content
182 in their feathers than species that feed on krill and squid (Carravieri *et al.* 2013; Brasso *et al.* 2015).

183 The mercury content we found in the feathers of the Humboldt penguins was in the range of the
184 mercury levels found in penguins that forage in environments with a moderate degree of pollution
185 (Dunlop *et al.* 2013; Finger *et al.* 2015), and was comparable to previous investigations in the
186 African penguin (Falkowska *et al.* 2013a; Squadrone *et al.* 2018) *ex-situ*. In these studies, Hg levels
187 in fish (the only source of food) were comparable, and in the range of 0.022-0.069 mg Kg⁻¹,
188 demonstrating an important bio-magnification of this metal in captive penguins, which were shown
189 to have Hg levels in their feathers in the range (mean values) of 2.0-2.8 mg Kg⁻¹.

190 Other metals that seem to bio-magnify in Humboldt penguins' feathers, presenting higher values

191 than in capelin (Table 1) were Al, Co, Cr, Cu, Mn, Ni, Pb, Se, Sn and Zn. Some of these non-
192 essential elements, such as aluminium and lead, are known to have an affinity to feathers and are
193 likely to bind to the sulfhydryl groups in keratin (Lucia *et al.* 2010; Sterner 2010), while other
194 elements, such as zinc, are essential in feather formation.

195 Arsenic was found in Humboldt penguin feathers at levels of an order of magnitude lower than in
196 fish (Table 1), in agreement with our previous investigation on *S. demersus* (Squadrone *et al.* 2018),
197 and comparable to concentrations found in wild penguins (Jerez *et al.* 2011; Finger *et al.* 2015).
198 This phenomenon whereby metal concentrations in tissues decreases with increasing trophic levels
199 is known as bio-dilution (Campbell *et al.*, 2005) or bio-minification (Pakrashi *et al.*, 2014) and was
200 observed here for arsenic (Table 1).

201 Cd, Fe and V presented comparable levels in fish and feathers (Table 1), which were in the range of
202 values found in penguins living in weakly contaminated environments (Jerez *et al.* 2011; Dunlop *et*
203 *al.* 2013; Finger *et al.* 2015).

204 Comparing our results with other investigations regarding seabirds (e.g. Burger and Gochfeld 2000
205 a, b; Becker 2003, Burger *et al.* 2009; Lucia *et al.* 2010; Becker *et al.* 2016), some general
206 considerations can be made on the transfer of metals from food to birds in marine food chains,
207 revealed by these analyses on feathers:

208 1. Different metal concentrations in seabirds are linked to differences in foraging, and
209 monitoring seabirds also gives the opportunity to assess levels of contamination in their
210 food, signalling possible “hot spots” of contamination.

211 2. Seabird feathers are particularly convenient for monitoring metal pollution in marine food
212 webs, allowing non-destructive sampling and retrospective studies; in particular, feathers are
213 an indicator of internal contamination especially for metals that have an affinity for the thiol
214 (SH) group in keratin, such as Hg and Pb (Furness and Camphuysen, 1997; Burger *et al.*
215 2009; Jakimska *et al.* 2011).

- 216 3. A clear relationship between levels of Hg and Cd in prey organisms and in seabirds was
217 usually observed, Hg is higher in feathers of fish-eating seabirds and Cd in squid-eating sea-
218 birds, even if feathers are not the tissues for Cd accumulation, which occurs instead in kid-
219 neys and bone after ingestion (Furness and Camphuysen, 1997).
- 220 4. Prey size also influenced Hg bioaccumulation; in fact levels were higher in seabirds that
221 feed on larger fish which contain higher Hg levels (Xavier and Croxall, 2007).
- 222 5. The uptake of Hg in seabirds is influenced by several factors in addition to diet (Monteiro *et al.*, 1998; Burger and Gochfeld, 2000 a, b), such as habitat and migration patterns (Carravieri *et al.*, 2014a).
- 225 6. Investigations regarding sex-related variations in seabirds' feathers has led to contrasting re-
226 sults, but gender specialization in feeding habitats, rather than physiological characteristics
227 of seabirds, seem to explain different Hg levels in male and females (Kojadinovic *et al.*
228 2007; Becker *et al.*, 2016; Carravieri *et al.*, 2014a).
- 229 7. High Hg levels in feathers are also related to a less efficient detoxification process due to re-
230 duced moult frequency, as feathers are the major pathway of Hg elimination (Becker *et al.*
231 2016).
- 232 8. The metalloid arsenic is subjected to bio-dilution through marine food chains, and lower
233 trophic marine animals show higher arsenic concentrations than higher trophic marine
234 animals (Campbell *et al.*, 2005; Pakrashi *et al.*, 2014); consequently As is found at lower
235 levels in seabirds feathers than in their prey.

236 The monitoring of chemicals by utilizing seabirds is of great importance to detect environmental
237 changes, but is also crucial for protecting birds in their environments and for planning measures
238 and strategies for environmental protection and bird conservation.

239 4.2 Comparison with a previous study on captive penguins (*Spheniscus demersus*)

240 We have not found any investigations regarding metal content in captive penguin feathers other than
241 our previous study on *Spheniscus demersus* from a North-western Italian facility (Squadrone *et al.*

2018) and the aforementioned study by Falkowska and co-authors (2013) reporting mercury concentrations in feathers of a colony of African penguins hosted in Gdansk Zoo (Poland). However, considering that zoos and aquaria contribute to the *ex-situ* conservation of a variety of endangered animal species, it is essential to control and minimize the exposure to contaminants of species maintained under human care, such as penguins.

In our investigations, the colonies of the two species of the genus *Spheniscus* hosted in Italian facilities, *S. humboldti*, (this study), and *S. demersus*, (previous investigation) were fed with one species of fish, respectively, capelin and herring. Despite the different fish species, metal levels in the penguins' food (Figure 1) were entirely comparable, with the exception of Cd and V which were found at low concentrations (0.054 and 0.052 mg Kg⁻¹) in capelin and < LOQ in herring (Squadrone *et al.* 2018); on the contrary, tin was found < LOQ in capelin and at low concentrations in herring (0.020 mg Kg⁻¹).

As a consequence, the two species belonging to the genus *Spheniscus* are exposed through their diet to very similar metal concentrations (Figure 1), apparently safe for penguins, as all metal levels in fish are not of particular concern, and are in any case, below the limits set by European Regulations for food and feed.

However, we found that metal concentrations in penguin feathers were very different in the two species, as shown in Figure 2. All metals, with the exception of As and Hg, were found at higher concentrations ($p < 0.01$) in *S. demersus* than in *S. humboldti*.

The relationship between Hg and As feather concentrations and the relative proportions of these metals in the penguins' food are obvious, but different conclusions can be made for the other trace elements. Although we may consider that the differences could be linked to a different accumulation in the two species of *Spheniscus*, it is also likely that the different way in which these penguins are bred in captivity could affect their body burden of metals. The Humboldt penguin colony of this study is kept indoors in a zoological facility, while the African penguin colony of our previous investigations is kept in an outdoor communal exhibit of another facility that is located close to one

268 of the most industrialized areas of Northern Italy. The levels of Mn, Ni, Cr in these African
269 penguins' feathers were in the range of concentrations recorded in wild birds living in industrialized
270 and polluted areas (Burger and Gochfeld, 2009; Abdullah *et al.* 2015; Squadrone *et al.* 2018).

271 Studying these metals in penguin feathers is particularly interesting, as these metals are directly
272 connected to several human contaminant activities. Studies performed in Antarctica found that these
273 elements are more abundant in penguin feathers from areas characterized by a major human
274 presence (e.g. Jerez *et al.* 2011), and higher metal concentrations have been attributed to the local
275 pollution of foraging areas.

276 In captive penguins, exposure by diet was comparable in the two species we considered, but the
277 Humboldt penguin colony living indoors seemed to be somehow protected by other sources of
278 anthropogenic exposure compared to the African penguin colony hosted outdoors, which presented
279 a higher degree of metal levels in their feathers (Figure 2). These findings suggest that the
280 environmental availability of metals in captive seabirds could depend on many factors in addition to
281 diet and that the environment in which penguins live should be carefully controlled.

282

283 Further investigations should be carried out in zoo facilities and in their surrounding areas, to verify
284 the potential presence of non-conventional sources of metal exposure in addition to diet; moreover,
285 it could be of particular interest to analyse penguins of different species kept in the same conditions
286 in zoos and aquaria for a better understanding of accumulation of metals.

287

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292 *Ethical statement* - This research conformed to the Ethical Guidelines for the Conduct of Research
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Figure 1 Trace elements concentrations in capelin (Humboldt penguin's food) and herring (African penguin's food (mg Kg^{-1} log scale).

Figure 2 Box-plots with trace elements concentrations (mg Kg^{-1} , mean $\pm$ SD) in feathers of African penguin (Zoom Biopark, Torino, Italy) and Humboldt penguin (Acquario di Cattolica, Rimini, Italy).

Table 1. Trace element concentrations (mean $\pm$ SD, mg kg⁻¹) in feathers and food of a *Spheniscus humboldti* colony.

Element	Feathers	Capelin
Al	12 $\pm$ 1.8	0.21 $\pm$ 0.052
As	0.14 $\pm$ 0.060	1.8 $\pm$ 0.22
Cd	0.060 $\pm$ 0.035	0.054 $\pm$ 0.012
Co	0.027 $\pm$ 0.010	<0.010
Cr	0.54 $\pm$ 0.014	0.012 $\pm$ 0.005
Cu	11 $\pm$ 4.5	0.85 $\pm$ 0.32
Fe	17 $\pm$ 2.1	11 $\pm$ 2.5
Hg	2.8 $\pm$ 0.28	0.022 $\pm$ 0.006
Mn	2.6 $\pm$ 0.42	0.42 $\pm$ 0.032
Ni	0.78 $\pm$ 0.23	0.076 $\pm$ 0.008
Pb	0.18 $\pm$ 0.05	0.010 $\pm$ 0.003
Se	0.85 $\pm$ 0.12	0.26 $\pm$ 0.011
Sn	0.031 $\pm$ 0.010	<0.010
V	0.076 $\pm$ 0.011	0.052 $\pm$ 0.021
Zn	51 $\pm$ 7.2	7.2 $\pm$ 1.1