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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 Valparaiso 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 (Burger 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.

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 m^2, including a salt-water tank of 35 m^2 (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 ultrawave oven (ETHOS 1, Milestone,) with 7 mL of HNO_3 (70% v/v) and 1.5 mL of H_2O_2 (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

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

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

3. Results

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

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

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

4. Discussion

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

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

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

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

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

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

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

Other metals that seem to bio-magnify in Humboldt penguins' feathers, presenting higher values than in capelin (Table 1) were Al, Co, Cr, Cu, Mn, Ni, Pb, Se, Sn and Zn. Some of these non-essential elements, such as aluminium and lead, are known to have an affinity to feathers and are likely to bind to the sulfhydryl groups in keratin (Lucia *et al.* 2010; Sterner 2010), while other elements, such as zinc, are essential in feather formation.

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

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

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

1. Different metal concentrations in seabirds are linked to differences in foraging, and monitoring seabirds also gives the opportunity to assess levels of contamination in their food, signalling possible “hot spots” of contamination.
2. Seabird feathers are particularly convenient for monitoring metal pollution in marine food webs, allowing non-destructive sampling and retrospective studies; in particular, feathers are an indicator of internal contamination especially for metals that have an affinity for the thiol (SH) group in keratin, such as Hg and Pb (Furness and Camphuysen, 1997; Burger *et al.* 2009; Jakimska *et al*. 2011).

3. A clear relationship between levels of Hg and Cd in prey organisms and in seabirds was usually observed, Hg is higher in feathers of fish-eating seabirds and Cd in squid-eating seabirds, even if feathers are not the tissues for Cd accumulation, which occurs instead in kidneys and bone after ingestion (Furness and Camphuysen, 1997).
4. Prey size also influenced Hg bioaccumulation; in fact levels were higher in seabirds that feed on larger fish which contain higher Hg levels (Xavier and Croxall, 2007).
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).
6. Investigations regarding sex-related variations in seabirds' feathers has led to contrasting results, but gender specialization in feeding habitats, rather than physiological characteristics of seabirds, seem to explain different Hg levels in male and females (Kojadinovic *et al.* 2007; Becker *et al.,* 2016; Carravieri *et al.,* 2014a).
7. High Hg levels in feathers are also related to a less efficient detoxification process due to reduced moult frequency, as feathers are the major pathway of Hg elimination (Becker *et al.* 2016).
8. The metalloid arsenic is subjected to bio-dilution through marine food chains, and lower trophic marine animals show higher arsenic concentrations than higher trophic marine animals (Campbell *et al*., 2005; Pakrashi *et al.,* 2014); consequently As is found at lower levels in seabirds feathers than in their prey.

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

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

We have not found any investigations regarding metal content in captive penguin feathers other than 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 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 of the most industrialized areas of Northern Italy. The levels of Mn, Ni, Cr in these African penguins' feathers were in the range of concentrations recorded in wild birds living in industrialized and polluted areas (Burger and Gochfeld, 2009; Abdullah *et al.* 2015; Squadrone *et al.* 2018). Studying these metals in penguin feathers is particularly interesting, as these metals are directly connected to several human contaminant activities. Studies performed in Antarctica found that these elements are more abundant in penguin feathers from areas characterized by a major human presence (e.g. Jerez *et al.* 2011), and higher metal concentrations have been attributed to the local pollution of foraging areas.

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

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

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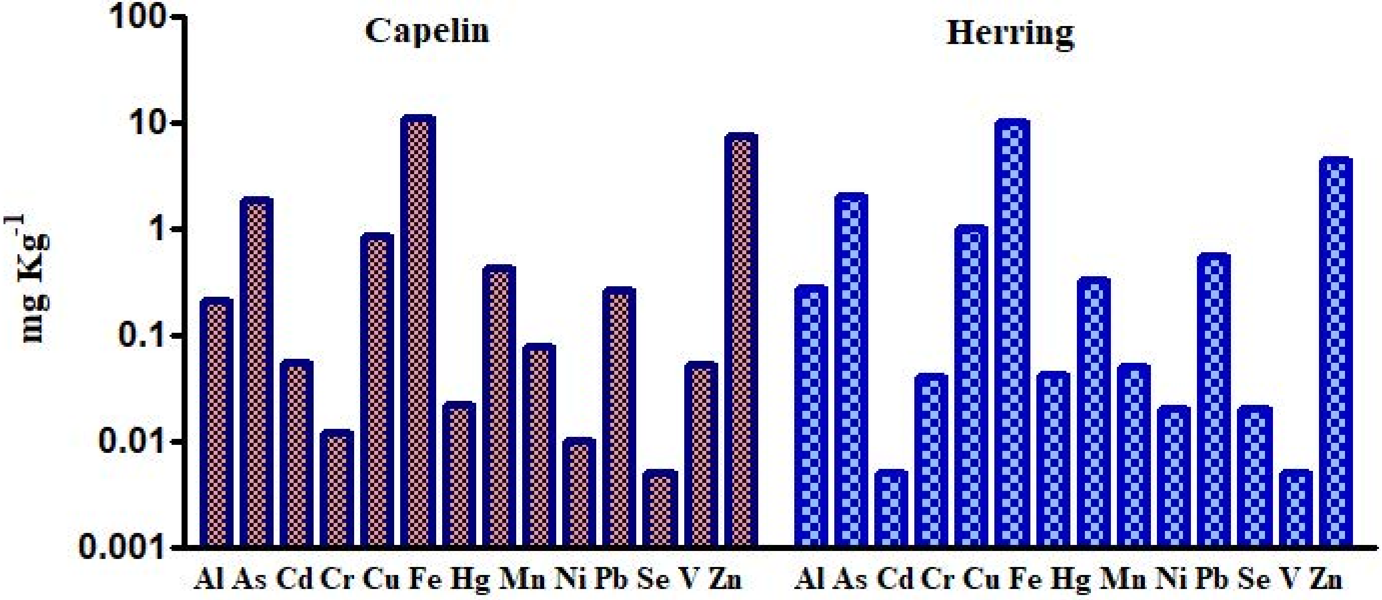
Figure 1 Trace elements concentrations in capelin (Humboldt penguin's food) and herring (African penguin's food (mg Kg^{-1} log scale).

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


Capelin
Herring
mg Kg-1
100
10
1
0.1
0.01
0.001
Al As Cd Cr Cu Fe Hg Mn Ni Pb Se V Zn
Al As Cd Cr Cu Fe Hg Mn Ni Pb Se V Zn

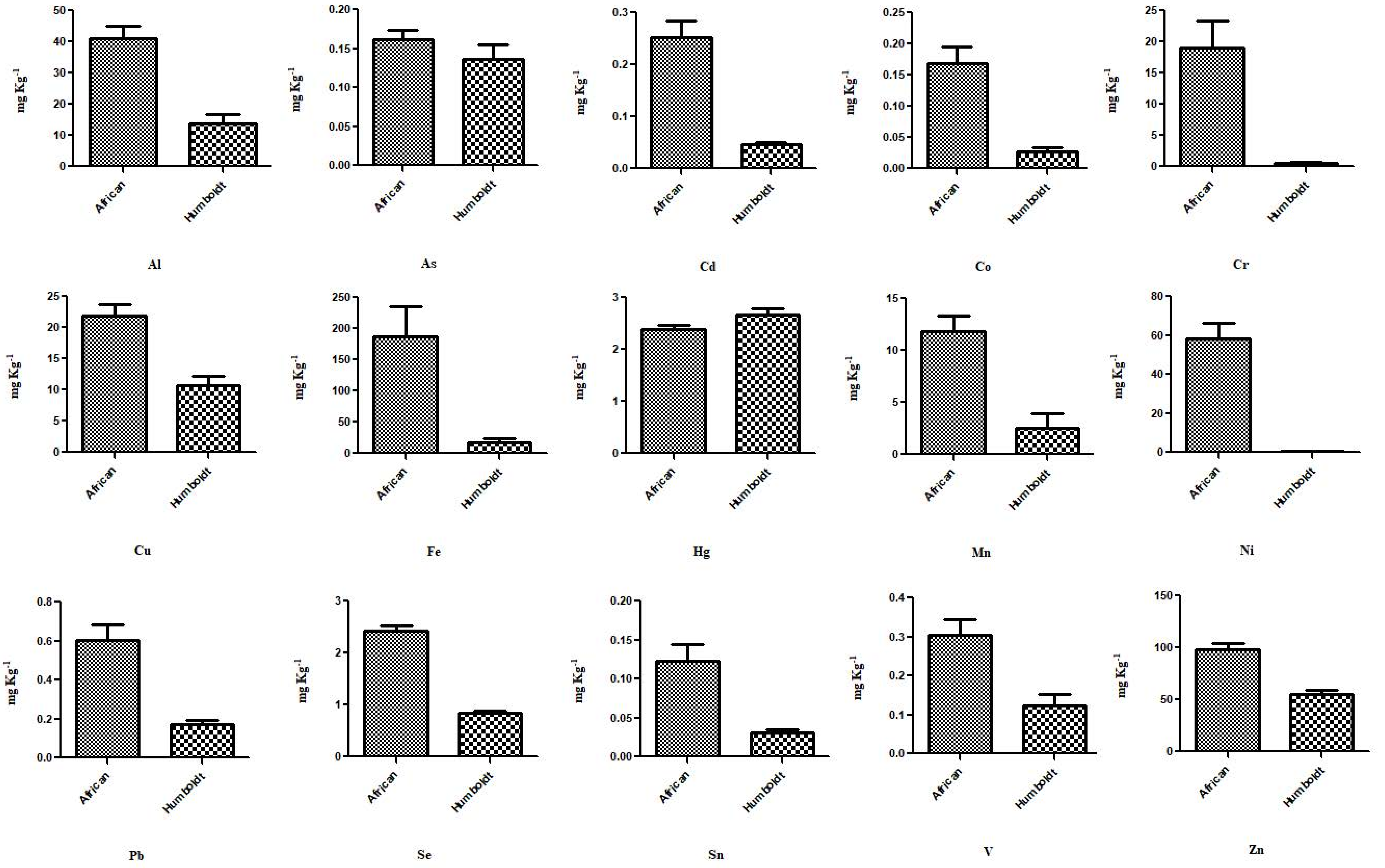

mg Kg-1
African
Humboldt
Al
As
Cd
Co
Cr
Cu
Fe
Hg
Mn
Ni
Pb
Se
Sn
V
Zn

Table 1. Trace element concentrations (mean ± SD, mg kg^{-1}) in feathers and food of a *Spheniscus humboldti* colony.

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