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SHORT COMMUNICATION

First report of metals and metalloids on bone and claw tissues of Humboldt penguins (*Spheniscus humboldti*)

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ABSTRACT. Samples of bones (humerus) and claws of adult Humboldt penguins (*Spheniscus humboldti*) were opportunistically obtained from twenty-seven carcasses at two important nesting sites in northern Chile: Chañaral Island (CHI) and Pan de Azúcar Island (PAI). The concentrations of trace elements (Cu, Zn, Pb, Ni, Fe, Se, As, Br, Mn and Cr) were determined by X-ray fluorescence. The highest levels (mean ± standard deviation, μg/g dry weight) of Cu (26.57 ± 4.08), Zn (163.9 ± 42.7), Pb (1.86 ± 1.53), Ni (0.31 ± 0.03), Se (7.70 ± 4.87) and Cr (0.25 ± 0.24) were detected in bones, whereas the highest levels of Fe (3,162 ± 1,579), As (6.75 ± 4.21), Br (0.12 ± 0.06) and Mn (76.7 ± 47.9) were found in claws. In bones, Se and Ni levels were higher ($P < 0.05$) in CHI than in PAI. In claws, the contents of Pb, Fe, and Mn were higher at CHI than those at PAI, whereas only As exhibited higher contents at PAI than those found at CHI. The trace element content in the claws and bones found herein may be the result of either acute or chronic exposure to penguins, respectively. These findings may serve as a baseline for further studies to design adequate and opportune plans to protect this vulnerable species.

Keywords: Seabirds; trace elements; marine pollution; northern Chile; X-ray fluorescence.

INTRODUCTION

The Humboldt penguin (*Spheniscus humboldti*) naturally resides along the Pacific coast of South America, from Perú to Chile (De la Puente *et al*., 2013), with 23,800 mature individuals. In Chile, this species inhabits between Arica and Chiloé in colonies that are mostly concentrated between 21°S and 34°S (Wallace & Araya, 2015), with a population that ranged from 40,000 to 47,000 in the past (Schlosser *et al*., 2009), and has shown a remarkable decline, with a population of less than 5,000 pairs, where the presence of polluting waste is mentioned among the greatest threats to the species (Simeone *et al*., 2018).

Because it is a species classified as vulnerable by the IUCN Red List of Threatened Species in 2020 (https://www.iucnredlist.org), it is extremely difficult to obtain samples such as blood or internal organs, which are considered invasive to birds. Studies focusing on the assessment of metal contamination in the bones and claws of penguins remain quite limited because these birds are protected by law, and thus, these samples can only be obtained in cases where opportunistically dead bodies are available.

Trace elements can have toxic effects on living organisms (Rodríguez & Mandalunis, 2018). Heavy metals can occur naturally (e.g. volcans) at low levels in the environment, and in larger amounts, they can impact health (Newman, 2015). The occurrence of trace elements in aquatic ecosystems is mainly due to anthropogenic activities such as antifouling coatings, sewage discharge, waste incineration, coal combustion, oil spills, pipe corrosion, and solid waste disposal (Bargagli, 2000; Caccia *et al*., 2003; Duruibe *et al*., 2007; Zhang & Ma, 2011). In northern Chile, the massive development of mining activities has had a detrimental effect on coastal ecosystems due to elevated metal concentrations (Ramirez *et al*., 2005; Stauber *et al*., 2005). Numerous industries (e.g., mining, fishing, seaports) and cities (e.g., Arica, Iquique, Antofagasta, Chañaral) in northern Chile discharge waste materials into the sea, leading to increased levels of certain metals that are considered hazardous to ecosystem health and biota (Vermeer & Castilla, 1991; Cortés & Luna-Jorquera, 2011).

Metal (loids) such as Pb, Cu, Fe, Mn, As, Ni and Zn may be sequestered in bones, which become a concern in animal's health as these metals can interfere with calcium homeostasis, inhibit bone-forming cells (osteoblasts), stimulate bone absorption cells (osteoclasts) and alter the mineralization process. This can lead to decreased bone density, increased risk of fractures and impaired skeletal development in growing animals (Newman, 2015; Rodríguez & Mandalunis, 2018; Ciosek *et al*., 2023). Non-essential elements (e.g., Pb, Cd, Ag, Ti, and As) are extremely toxic with no biological functions, whereas essential elements (e.g., Cu, Fe, Cr, Se, Br, Ni, Zn and Mn) are required in very small amounts because they perform vital functions for the maintenance of animal life (McCall *et al*., 2014; Nordberg & Nordberg, 2016). Some metals (e.g. Pb, Cu, Cr, Ni, and Zn) and metalloids (e.g. As) can induce severe adverse effects in birds, even an increase in the mortality rate (Newman, 2015). Commonly, Zn accumulation in birds has been linked to binding to metallothionein, but it can also accumulate in the muscles and bones (Wastney *et al*., 2000). Some less-known metals, such as Mn, can accumulate in the bone, cartilage, and tissues that are dense with mitochondria, and overexposure to Mn can cause serious health problems in birds (Sarnowski & Kellam, 2023). At high concentrations in biota, Ni is both toxic and carcinogenic (Newman, 2015), and wild birds from polluted environments can accumulate in bone higher Ni concentrations than other internal tissues (Outridge & Scheuhammer, 1993). Adverse effects of Se (a non-metal) in wild aquatic birds have been linked to pollution of the aquatic environment by anthropogenic activities, including impaired reproduction, reduced growth, histopathological lesions, among others (Hoffman, 2002). There is no available data on the non-metal Br levels in avian wildlife, but some evidence indicates that Br can alter the metabolism of rats and broilers with subsequent deleterious effects (Du Toit & Casey, 2010; Pavelka, 2004).

Human nails have been shown to be valuable biomarkers for heavy metal exposure, providing insight into long-term exposure due to their slow and continuous growth (Sukumar & Subramanian, 2007). Other studies have revealed that elements such as As, Cd, Hg and Pb accumulate in nails, reflecting both environmental and dietary exposure (Gualllar *et al*., 2002; Shokoohi *et al*., 2022). In birds, claws are similar to human nails, making them a useful bioindicator of heavy metal contamination. Mercury (Hg) concentrations in the talons of bald eagles (*Haliaeetus leucocephalus*) correlated significantly with levels in other tissues, suggesting that talons may serve as a non-lethal alternative for contaminant monitoring (Hopkins *et al*., 2007).

Despite the potential risks posed by metal contamination to seabirds, to the best of our knowledge, no studies have been conducted on bones or claws of Humboldt penguins. Consequently, the primary goals of this study were to evaluate, for the first time, the concentrations of selected trace elements in adult Humboldt penguins and to compare these levels with those in the existing literature. By acquainting these objectives, this work contributes to filling a gap in trace element accumulation in the claw and bone of Humboldt penguins, adding light to the potential risks to this vulnerable species and their habitats.

MATERIALS AND METHODS

Bone and claw samples of Humboldt penguin carcasses were collected opportunistically from Pan de Azucar and Chañaral Islands (Figure S1, Supplementary material). Pan de Azúcar Island, situated 1km away from the coastline is a site on the northern coast of Chile highly impacted by mining activities (Celis *et al*., 2014), whereas Chañaral Island is an isolated place, with little anthropogenic influence, 7 km away from the coastline and 100 km north of Coquimbo Bay. Both islands are important sites where Humboldt penguins nest. Bone (left femur) and claw samples of adult Humboldt penguin carcasses were carefully and opportunistically collected in December 2015 (nesting period of the species). Disposable plastic gloves were used for handling and storing samples. The bones (n=27) and claws (n=27) were collected from multiple individuals across the penguin colonies, as it was not feasible to assess metal contamination in each individual separately, but rather as a collection of that species. To maintain sample integrity, clean plastic containers and sealed plastic bags (Ziploc®) were used, where each sample was stored separately to avoid cross-contamination between samples.

First, the bones and claws were cleaned with alcohol swabs to ensure the removal of any external contamination (Mateo-Lomba *et al*., 2022). After cleaning, the samples were thoroughly rinsed (Milli-Q water) and left to dry at room temperature before element analysis.

Recently, low-cost X-ray fluorescence (XRF) has been used in wildlife and ecotoxicological studies with promissory results (Specht *et al*., 2019; Hampton *et al*., 2021; Celis *et al*., 2022). Thus, a portable XRF Niton XL3t GOLDD+ (Thermo-Fisher Scientific, Omaha, USA) was used to determine the burden of chemical elements in the samples. The blank was a certified 99.99% silicon dioxide (SiO2) analysed for every 20 samples. Precision and accuracy were verified using international reference standards Rare Earth Ore "CGL 124" (USZ-42 Mongolia Central Geological Laboratory), with precision > 98 % and accuracy within 95–99 %. The QA/QC detection limits (μg/g) were as follows: 1.43 for Cu, 8.72 for Zn, 0.26 for Pb, 0.07 for Ni, 23.52 for Fe, 0.39 for Se, 9.27 for As, 0.07 for Br, 0.04 for Mn and 0.16 for Cr. The uncertainty of the measurements (μg/g) were for bones as follows: Cu (1.08 ± 0.35), Zn (9.37 ± 3.01), Pb (1.47 ± 1.30), Ni (0.06 ± 0.01), Fe (105.96 ± 356.71), Se (4.15 ± 8.08), As (6.82 ± 5.28), Br (0.0024 ± 0.0014), Mn (0.13 ± 256.71) and Cr (0.43 ± 0.42); for claws were Cu (0.84 ± 0.10), Zn (6.08 ± 3.98), Pb (0.40 ± 0.38), Ni (0.04 ± 0.01), Fe (34.38 ± 36.52), Se (2.09 ± 6.23), As (9.12 ± 6.44), Br (0.004 ± 0.002), Mn (0.07 ± 0.11) and Cr (0.15 ± 0.10).

Nonparametric statistical methodologies were employed because the data did not meet the assumptions of normality and homoscedasticity even after applying a log transformation. The differences among the element levels were determined using Kruskal-Wallis analysis. Post hoc tests were conducted with critical differences in the mean rank. To estimate the relationship between the trace element concentrations in the bones and claws, ordinary least squares regression was used. The uncertainty value (σ) for the portable XRF was determined using the following equation: $\sigma = (C/NET)\,(BGK/t)^{0.5}$, where C represents the element concentration, BKG is the estimated background count obtained through fitting, t is the measurement time, and NET is the net element count derived from the Gaussian function in the fitting process (Zhang *et al*., 2021). For bones and claws, Spearman rank correlation coefficients were calculated to determine the relationship between the trace element levels in bones and claws. Statistical significance was set at $P < 0.05$. The data were analysed (t-Student) using SPSS 27.0.

RESULTS AND DISCUSSION

Information on the levels of trace elements in Humboldt penguins is fragmentary; only a few studies have focused on the bones of penguins worldwide, whereas no studies have been conducted on penguin claws (Espejo *et al*., 2017). The concentrations of Cu, Zn, Pb, Ni, Fe, Se, As, Br, Mn and Cr are shown in Table 1, which were detected in both matrices and locations studied. The highest levels of Cu, Zn, Pb, Ni, Se and Cr were detected in bones, whereas the highest contents of Fe, As, Br and Mn were found in claws. The highest mean concentrations detected corresponded to Fe (3,162.2 µg/g) at Chañaral Island (CHI), whereas Br exhibited the lowest levels (0.05 µg/g) at Pan de Azúcar Island (PAI). In bones, the levels of Se and Ni were significantly higher at CHI than those levels found at PAI, whereas Cu, Zn, Pb, Fe, As, Br, Mn and Cr contents showed no statistical differences. In the claws, the levels of Pb, Fe, and Mn were higher in CHI than in PAI ($P < 0.05$), whereas As values were not ($P < 0.05$, Table 1).

At PAI the levels of Zn, Pb and Mn in the bones were higher than those levels found in the claws, while As and Br contents showed the opposite ($P < 0.05$). At CHI, the concentrations of Cu, Ni and Se found in the bones were statistically higher than those detected in the claws, but Fe levels were higher in claws than in bones ($P < 0.05$). These findings in bones and claws of this species from the two studied geographical areas may be linked to different anthropogenic sources, since Pan de Azúcar Island presents major human activities (e.g. mining, industries) than Chañaral Island (Celis *et al*., 2014).

Significant relationships were found between the concentrations of the trace elements (Figure 1). In both bone and claw samples, a positive correlation was noted between Cu-Zn, Cu-Ni, Cu-As, Zn-Se, Fe-Mn, Fe-Cr, Se-As and Mn-Cr ($P < 0.05$). Only in bones was a significant positive correlation noted for Cu, Zn, and Ni with Cr, and for Ni with Pb, Fe, and Mn. Similar findings were noted for Se with Cu, Pb, Ni, Zn-As, and As with Cu in the claws (Figure 1). Similarly, Squadrone *et al*. (2018) observed a positive correlation between Cu-Ni and Fe-Cr in feathers of African penguins (*Spheniscus demersus*). Another study by Celis *et al*. (2015) reported a significant positive correlation between Cu-As, Zn-As and Cu-Zn in excreta of Adélie penguins (*Pygoscelis adeliae*) from Antarctica. Probably, the positive correlations indicate that the elements for all the study colonies are of the same source. On the other hand, negative correlations ($P < 0.05$) were found between Cu-Br, Ni-Br, Fe-Se, As-Mn and Se-Mn, in both bones and claws.

The contents of As, Cu, Pb, Mn, Se and Cr in the bones found herein are higher, the Ni contents are lower, while the Zn levels are within the range of those levels detected in bones of penguins of the genus *Pygoscelis* from different locations of Antarctica (Table S1, Supplementary material). The comparison with others aquatic birds revealed that our Ni and Pb levels in bones are lower than Ni (10.5-36.1 µg/g dw) and Pb (32.4-59 µg/g dw) levels, whereas our Cu levels are higher than Cu (4-8.2 µg/g dw) levels reported by van Eeden

Table 1.
Concentrations of chemical elements (µg/g, d.w) in bones and claws of Humboldt penguins sampled at nesting sites from Pan de Azúcar Island (PAI, n=23) and Chañaral Island (CHI, n=4).

	Bones		Claws	
	PAI	**CHI**	**PAI**	**CHI**
Cu	23.17 ± 6.80 a	26.57 ± 4.08 a *	21.50 ± 5.76 a	17.18 ± 2.23 a **
Zn	163.9 ± 42.7 a *	159.0 ± 26.7 a	128.3 ± 69.9 a **	130.6 ± 72.1 a
Pb	1.86 ± 1.53 a *	1.64 ± 0.42 a	0.49 ± 0.39 a **	1.23 ± 0.62 b
Ni	0.27 ± 0.03 a	0.31 ± 0.03 b *	0.22 ± 0.04 a	0.26 ± 0.03 a **
Fe	2,247.1 ± 2,281.6 a	1,272.3 ± 566.1 a *	1,361.8 ± 1,037.3 a	3,162.2 ± 1,579.1 b **
Se	1.95 ± 5.05 a	7.70 ± 4.87 b *	2.53 ± 5.5 a	0.38 ± 3.05 a **
As	3.53 ± 3.01 a *	4.33 ± 1.31 a	6.75 ± 4.21 a **	1.98 ± 1.83 b
Br	0.05 ± 0.03 a *	0.08 ± 0.05 a	0.12 ± 0.06 a **	0.09 ± 0.04 a
Mn	45.1 ± 53.4 a *	30.57 ± 14.93 a	19.86 ± 32.35 a **	76.7 ± 47.9 b
Cr	0.25 ± 0.24 a	0.23 ± 0.06 a	0.14 ± 0.08 a	0.24 ± 0.11 a

Data are shown as mean ± standard deviation. Different letters between collecting sites for the same biological matrix indicate significance at $P < 0.05$. Differences between bones and claws for the same location are indicated with an asterisk ($P < 0.05$).

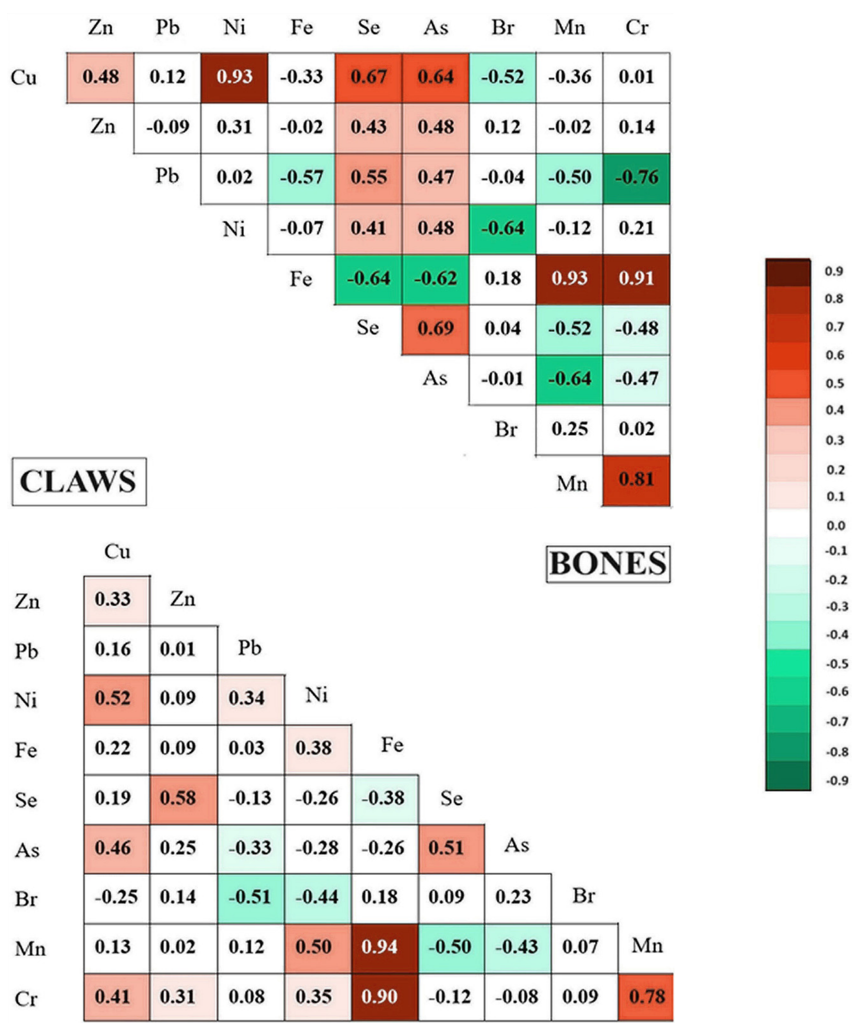

Figure 1.
Correlation matrix among the concentrations of selected trace elements.

and Schoonbee (1996) in bird bones from a metal-polluted wetland in South Africa. Our mean Fe levels in bones were 79.4 times higher than the mean Fe levels measured in the bone (tarsometatarsus) of diving ducks (*Aythya ferina*) from the Baltic Sea (Kalisinska *et al*., 2007). The higher As, Cu, Pb, Mn, Se and Cr concentrations in bones detected herein could be explained by the major copper-mining activities occurring in northern Chile (Vermeer & Castilla, 1991; Ramírez *et al*., 2005; Cortés & Luna-Jorquera, 2011), where the nesting sites focus of this study are located.

Pollutants in bones accumulate during the lifetime of the organism; therefore, metals in Humboldt penguin bones may be considered an indicator of long-term exposure, as stated by Barbosa *et al*. (2013) in Antarctic penguins. Our Pb levels are < 10 µg/g dw, a threshold known to be toxic to birds (Scheuhammer, 1987), which suggests very small biological effects in the Humboldt penguin. However, there was a difference between Pb levels in penguins from Chañaral Island (a colony with almost no contact with humans) and penguins from Pan de Azúcar Island (a site with mining and human presence), possibly due to the indirect effects of human impact on Humboldt penguins. Metal (loid) intoxication has a negative impact on human health, as long/short term exposure to high Zn, As, Cu, Cr, Pb, Ni, and Fe concentrations alters the bone remodelling process, leading to the development of different bone pathologies (Rodríguez & Mandalunis, 2018). Chronic exposure to metals may pose a threat to penguins (Espejo *et al*., 2017a) and humans (Newman, 2015). When an organism is exposed to metal contamination, claws tend to accumulate trace elements from six months to 1.5 years, whereas bones are more representative in terms of years or even decades of exposure (Rabinowitz, 1991; Gutiérrez-González *et al*., 2019). The accumulation of metal (loids) in the bone, although not causing any problems, can trigger the reappearance of chronic toxicity by mobilisation of these elements in the body (Silbergeld *et al*., 1988). The data on bones and claws found herein can be useful for future research to determine if there are differences in the temporality of exposure and accumulation dynamics. Trends observed in claws and bones over time would result from either acute or chronic exposure to penguins, respectively, an issue that needs more attention.

Studies on trace metals in vertebrate animals are useful for extrapolating their potential effects in humans (Newman, 2015). It is estimated that there will be an increasing demand for trace elements in the manufacture of new products (e.g. solar panels, wind turbines, and electronic devices) in the near future, with a 300% increase in the demand for chemical elements such as Pb and Ni, among others (Erel *et al*., 2021). This raises the concern that the increasing use of various toxic metals may result in high concentrations in humans, predominantly in populations that are not fortunate enough to live in regulated and monitored regions. For this reason, the increased use of metals must be accompanied by the maximum recycling of metals and consideration of environmental and toxicological aspects in the selection of metals for industrial use (Babayigit *et al*., 2018). This study proved that penguin bone may be used to monitor trace element contamination in aquatic ecosystems. Further research is required in this regard.

In conclusion, this study adds novel data regarding the accumulation of trace elements in penguin bones and it is the first study to measure the levels of trace elements in penguin claws. Most of the elements studied herein in the bones of Humboldt penguins were higher than those previously reported in bones of different penguin species elsewhere. Our findings add valuable data on trace element accumulation in the Humboldt penguin on the northern coast of Chile. Also, this study proved that X-ray fluorescence is a useful low-cost analytical procedure for assessing trace elements in the bones and claws of penguins. Because claws are a continuously growing tissue, the question of whether claws can provide reliable spatial and temporal data on trace metal contamination in penguins is an issue that needs to be further investigated. Considering that Humboldt penguins are vulnerable species which face a dramatic population decline, this study adds valuable information that can help to elucidate whether metal contamination affects this species. Further studies are needed to better understand any possible cause in order to be able to implement measures to reverse such a decline.

DECLARATIONS

Competing interest statement

The authors declare that they have no conflicts of interest.

Ethics statement

This study was approved by the Research and Animal Ethics Committee of the Faculty of Veterinary Sciences, Universidad de Concepción, Chile.

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Author contributions

Adesina provided the concepts, data analysis, and writing; Espejo worked with data collection and analysis; Celis worked with writing of the manuscript; Sandoval collaborated with data analysis; Specht revised the manuscript and analyzed the data.

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