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Evaluation of Metal Partitioning across Humboldt Penguin (*Spheniscus humboldti*) Egg Components

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ABSTRACT: Humboldt Penguin (*Spheniscus humboldti*) population declines are attributable to several multifaceted anthropogenic impacts. At present, the exposure of Humboldt Penguins to high concentrations of heavy metals in the marine environment is a preeminent concern, due to mining along the Peruvian coast near key rookery sites. Metal and selenium concentrations were determined in eggs collected from September 2020 to April 2021 from a managed-care penguin population at the Brookfield Zoo to establish reference values for health indices conducted on wild populations. Concentrations of 16 elements, with emphasis on those found in mine efflux—arsenic, cadmium, copper, lead, mercury, selenium, and zinc—were assessed via inductively coupled plasma mass spectrometry in yolk, albumen, and eggshell. Data analyses indicate a clear delineation between egg constituents, with lipid-rich yolk displaying notably higher concentrations (μg/g) of arsenic (0.20 ± 0.064), chromium (0.086 ± 0.03), cobalt (0.01 ± 0.003), iron (238.65 ± 54.72), lead (0.32 ± 0.97), manganese (2.71 ± 0.66), molybdenum (0.57 ± 0.14), tin (3.29 ± 0.99), and zinc (64.03 ± 13.01) than other components (albumen and eggshell). These data confirm that heavy metals are partitioned differently across Humboldt Penguin egg components, which provides insight into the potential connection between embryonic nutrient source contamination and subsequent chick viability.

Key words: Albumen, eggshell, heavy metals, Humboldt Penguin, yolk.

INTRODUCTION

Seabirds play an integral role in the establishment and preservation of stable marine ecosystems through their extensive trophic diversity and global ubiquity (Colabuono et al. 2016). However, their populations are often destabilized by anthropogenic activities. The Humboldt Penguin (*Spheniscus humboldti*) has been recognized for nearly 2 decades as a species vulnerable to extinction, largely because of human activity (BirdLife International 2020). Population declines throughout the species' range of Peru and Chile are attributed to guano harvesting (Duffy 1983; Paredes and Zavalaga 2001), resource competition from commercial fisheries (Thiel et al. 2007), trophic restructuring following spatiotemporally cyclical El Niño Southern Oscillation events (Taylor et al. 2008), and industrial mining (Zavalaga and Paredes 1997). Although coastal mining operations have long threatened the species with habitat degradation and direct anthropogenic disturbance, including mass culls organized to remove the "nuisance" birds (Zavalaga and Paredes 1997; Paredes et al. 2002, 2003), recent mining expansions near the species' largest Peruvian rookery, Punta San Juan Reserve (PSJ, 15°22′S, 75°11′W), have made heavy metal exposure a significant concern (Adkesson et al. 2019).

Peru is the world's second largest producer of copper (Cu), zinc (Zn), and silver (Ag); it is also South America's leading contributor of gold (Au) and lead (Pb), third largest source of tin (Sn), and fourth largest source of molybdenum (Mo; Ministerio de Energía y Minas 2020). The country's largest open-pit iron (Fe) ore mine, producing 4 million tons annually, is within 20 km of PSJ, and an expanding copper mine is within 6 km (Adkesson et al. 2018).

Contaminants from local mines may reach the PSJ rookery directly via runoff from waste retention ponds where flocculated debris is consolidated before removal and disposal. Additionally, the northward flow of the Humboldt Current in the Pacific Ocean off the coast of South America may facilitate transport of contaminant runoff from Tacna, a mining community in southern Peru that manages the world's fifth largest copper mine, as well as copper tailings from dumping sites along Chile's northern coast (Castilla and Correa 1997).

Many metals, such as Cu, Fe, selenium (Se), and Zn, are essential micronutrients required to facilitate many physiologic processes; however, excess amounts, particularly of acutely toxic heavy metals, can lead to deleterious effects (Richards 1997). Heavy metals are a group of high-atomic-mass metals and metalloids that are toxic at low concentrations (Fergusson 1990), and previous research on Humboldt Penguins at PSJ confirmed heavy metal exposure in this protected population (Adkesson et al. 2019). Although heavy metal toxicity is dependent on dose, chemical form (e.g., organic vs. inorganic metal compounds), route of exposure, and physiologic parameters (e.g., sex, age, body condition, immune status), chronic exposure can cause severe physiologic detriment, including endocrine dysfunction, neurologic disruption, and immunohematologic complications (Scheuhammer 1987). Lipophilic metals, such as Cu, manganese (Mn), and Zn (Tjälve and Gottofrey 1991), exhibit distribution partiality once ingested and absorbed by the body, resulting in a disproportionate accumulation of contaminants in adipose-rich tissues, such as the brain. Metals also tend to accumulate in organs responsible for toxin elimination, such as the liver and kidneys (Das et al. 2003). Avian-specific effects of heavy metal exposure and bioaccumulation include decreased egg production, behavioral abnormalities (Fry 1995), and teratogenesis (Scheuhammer 1987).

Mechanisms of heavy metal elimination in birds include excretion (e.g., via feces), feather deposition, and egg offloading (Burger 1994; Ackerman et al. 2016, 2017, 2019). Although maternal offloading is well established as a physiologic pathway for toxin elimination in fowl (Holcman and Stibilj 1997; Grace and MacFarlane 2016; Kabeer et al. 2021), passerines (Mora 2003), and shorebirds (Burger 2002; Burger and Gochfeld 2004), heavy metal research on penguin egg components is scarce and, when present, almost entirely comprises data from Antarctic species (Metcheva et al. 2006, 2011; Smichowski et al. 2006; Jerez et al. 2011, 2013a, b; Celis et al. 2014). We aimed to quantify concentrations of heavy metals in the egg components (shell, yolk and white) of Humboldt Penguins, a South American species, using eggs from a managed-care population at the Brookfield Zoo, Chicago, Illinois, USA.

MATERIALS AND METHODS

Sample collection

Whole eggs were collected from nine breeding pairs of Humboldt Penguins under managed care at the Brookfield Zoo, Chicago, Illinois, USA, from September 2020 to April 2021. Eggs were refrigerated in plastic specimen containers (4 C) until shipment to Nova Southeastern University (Fort Lauderdale, Florida, USA) for analysis. Analyzed samples included eggshell (n=18), yolk (n=14), and albumen (n=14). Internal components for four eggs were omitted from analyses because of structural degradation.

Sample preparation and analysis

Whole eggs were rinsed with ultrapure deionized water (18.2 megohms) from a Barnstead water (Lake Balboa, California, USA) purification system, and gently scrubbed with a nylon cleaning brush. Eggs were then cracked along the equatorial axis and separated into eggshell, albumen, and yolk. Individual egg components were placed in acid-washed glass beakers and dried for a minimum of 24 h in an isotemp vacuum oven (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 100 C, with pressure reduced below 10^{-2} torr using a vacuum pump (14008-01 model Welch 1400 DuoSeal, Welch, Mt. Prospect, Illinois, USA). Approximately 0.2 g (dry mass) of each sample was placed in a microwave acid digestion vessel (Parr Instrument Company, Moline, Illinois, USA) with 4 mL of 30% hydrogen peroxide (Sigma-Aldrich, St. Louis, Missouri, USA) and 1 mL of concentrated trace-metal-grade nitric acid (Sigma-Aldrich). The vessel was heated in a conventional microwave oven at 400 W for 35 s to achieve internal device pressure up to 1200 psi and reactant temperatures up to 250 C, ensuring total sample digestion, then cooled and depressurized for 45 min.

Digested samples were shipped to the University of Southern Mississippi Center for Trace Analysis (Hattiesburg, Mississippi, USA) for analysis of 16 elements (15 metals plus selenium) via inductively coupled plasma-mass spectrometry (ICP-MS): aluminum (Al), arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), vanadium (V), and zinc (Zn). Before analysis, digested samples were diluted fivefold in 0.64 M ultrapure nitric acid containing 2 ppb indium as an internal standard and held in acid-washed Teflon autosampler vials (Thermo Fisher). Mass spectrometer scans were performed at low (Cd-111, Hg-199, 200, 201, 202, Pb-208), medium (Al-27, V-51, Cr-52, Mn-55, Fe-56, Co-59, Ni-60, Cu-63, Zn-66), and high (As-75, Se-77,82) resolution, depending on the isotope. To correct for molybdenum oxide interference on Cd, Mo-98 was monitored. External standards were used with a high standard and a blank rerun every eight samples. Two US Geological Survey reference water concentrations were also assessed as part of each analytical run to verify standardization. In several cases, sample calibration was also verified by standard additions. Blanks of ultrapure deionized water and trace metal basis nitric acid (3%, 4%, 5%) were used for quality control purposes. The ICP-MS detection limits of each element are provided in Supplementary Materials Table S1. No certified reference materials were used because none are available for heavy metal concentrations in egg components.

Statistical analyses were performed using Excel (version 16.60; Microsoft Corporation, Redmond, Washington, USA) and R statistical software (R 4.2.0 GUI 1.78 High Sierra build [8075]; R Core Team 2022). Geometric mean was used rather than arithmetic mean, to mitigate the impact of data outliers. A Shapiro-Wilk test was used to determine if the data were normally distributed, and a Bartlett's test was used to determine homogeneity of variances. Nonparametric analyses were used because of variable data distributions and lack of homogeneity. Kruskal-Wallis and post-hoc tests were performed to determine if metal concentrations varied among egg components. Potential correlations in heavy metal concentrations within egg components were assessed via Kendall's tau correlation (for $n<30$) and plotted as correlation matrices (Supplementary Materials Figs. S1–S3). Values below the detection limit (DL) were proxied as one-half of the DL. Statistical significance was identified when $P<0.05$. Selenium binds to organic Hg at a molar ratio of 1:1; therefore the molar ratio was calculated for each sample using

$$Molar\ Ratio = \frac{[\mathrm{Se}]/78.96\ \mathrm{g/mol}}{[\mathrm{Hg}]/200.59\ \mathrm{g/mol}},$$

where 78.96 g/mol and 200.59 g/mol represent the atomic masses of Se and Hg, respectively.

RESULTS

Summary statistics for egg component element data are presented by sample type (eggshell, albumen, and yolk) in Supplementary Materials Tables S2–S4. All 15 metals and Se were detected in yolk ($n=14$). Albumen ($n=14$) and eggshell ($n=18$) contained detectable levels of 13 of the 16 elements: Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se, Sn, V, and Zn. Arsenic, Hg, and Mo were below the detection limit in eggshells, and Mo could not be detected in albumen. Element distributions for each sample type (yolk, albumen, eggshell) are displayed in Fig. 1.

Molar Se:Hg ratios exceeded 1:1 for all yolk and albumen samples (Supplementary Materials Table S5). Mercury was not detected in eggshell, precluding molar Se:Hg ratio calculation for this component.

Yolk exhibited the highest geometric mean concentrations (μg/g) for As (0.20 ± 0.064), Co (0.01 ± 0.003), Cr (0.086 ± 0.03), Fe (238.65 ± 54.72), Mn (2.71 ± 0.66), Mo (0.57 ± 0.14), Pb (0.32 ± 0.97), Sn (3.29 ± 0.99), and Zn (64.03 ± 13.01; Table 1). Albumen had the highest concentrations of Al (4.19 ± 2.52), Cd (0.0009 ± 0.001), Cu (4.02 ± 1.22), Hg (0.62 ± 0.22), Se (1.52 ± 0.45), and V (0.0015 ± 0.03; Table 1). Eggshell had the highest concentration of Ni (0.31 ± 1.29; Table 1).

Statistically significant differences between eggshell and yolk occurred for all metals except

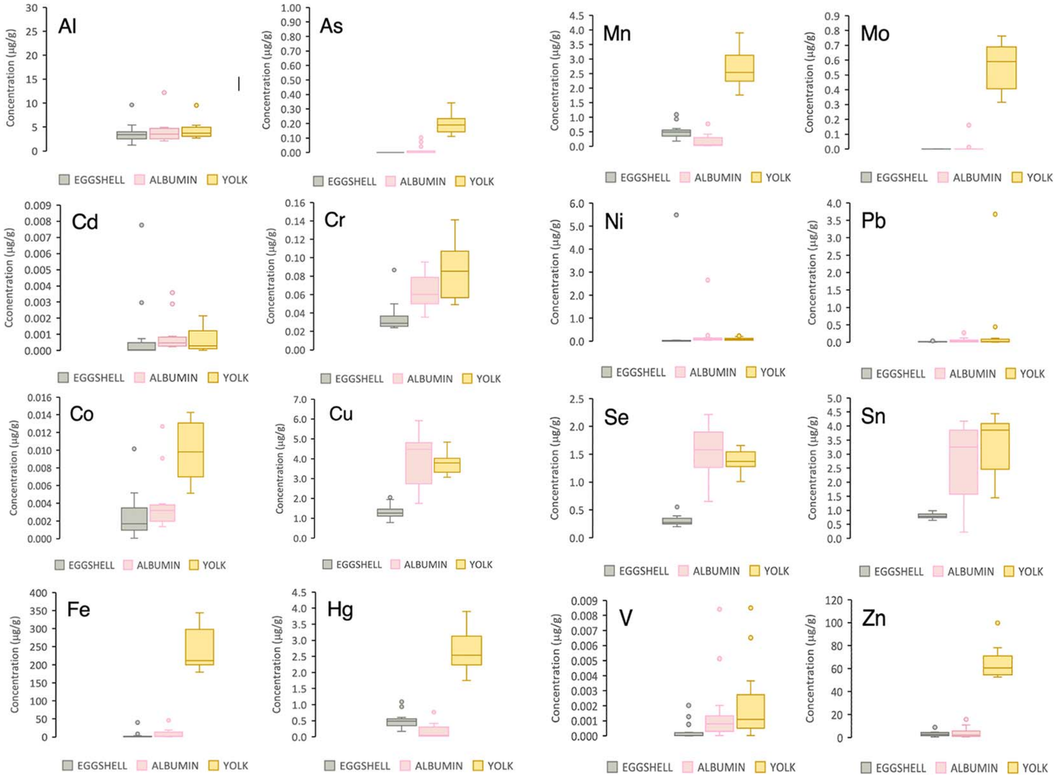

FIGURE 1. Geometric mean concentrations (µg/g) for aluminum (Al), arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), vanadium (V), and zinc (Zn) across egg component types for eggs from Humboldt Penguins (*Spheniscus humboldti*). Concentration data outliers are represented as circles above the maximum value.

Al, Cd, and Pb. Eggshell and albumen statistical differences were found for all metals except Al, As, Fe, Mo, and Zn. Arsenic, Co, Fe, Hg, Mn, Mo, and Zn concentrations differed significantly between yolk and albumen (Table 2). Aluminum was the only metal to show no variation among egg components.

Correlation matrices (Supplementary Materials Figs. S1–S3) represent associations between heavy metals within egg components. In yolk, highly positive ($r > 0.7$) correlations were observed between Se and Sn, Se and Mn, Hg and Ni, Cr and Ni, Cd and Ni, Fe and Zn, and Cu and Zn; highly negative correlations ($r > -0.7$) were observed between Sn and Mo, Ni and Mo, Mo and Hg, and Cr and Mo. Albumen had highly positive correlations between Zn and Mn, Zn and Fe, Sn and Cr, Mn and Fe, Pb and Co, Ni and Co, and Pb and Cd; highly negative correlations were not observed. Eggshell produced highly positive correlations between Se and Ni, V and Mn, Zn and Fe, Ni and Cd, and Pb and Al; highly negative correlations were not observed.

DISCUSSION

It is well established that wild penguins may act as reliable bioindicators of inorganic environmental pollution (Celis et al. 2014; Finger et al. 2015; Adkesson et al. 2018, 2019), but contaminant data are scarce from penguins in managed care. We therefore draw comparisons between our data and heavy metal concentrations reported for eggs and tissues from other bird species. The value of intertissue and interspecies heavy metal concentration comparisons in seabirds has been given by Ackerman et al.

TABLE 1. Summary of geometric mean and standard deviation for metal and selenium concentrations (μg/g) determined through inductively coupled plasma-mass spectrometry analysis of egg components of Humboldt Penguins (*Spheniscus humboldti*) from a managed-care population at the Brookfield Zoo, Chicago, Illinois, USA. The highest concentration for each element is denoted with boldface text.

	Sample type		
Element[a]	Eggshell	Albumen	Yolk
Al	3.61 ± 1.79	**4.19 ± 2.52**	3.86 ± 0.94
As	ND[b]	0.0164 ± 0.0328	**0.195 ± 0.064**
Cd	0.0007 ± 0.002	**0.0009 ± 0.001**	0.0006 ± 0.0007
Co	0.0024 ± 0.0023	0.0038 ± 0.0031	**0.0097 ± 0.003**
Cr	0.035 ± 0.015	0.063 ± 0.018	**0.0864 ± 0.031**
Cu	1.32 ± 0.33	**4.02 ± 1.22**	3.77 ± 0.52
Fe	4.04 ± 9.23	10.4 ± 16.03	**239 ± 54.7**
Hg	ND	**0.618 ± 0.217**	0.079 ± 0.061
Mn	0.498 ± 0.22	0.171 ± 0.219	**2.71 ± 0.66**
Mo	ND	ND	**0.575 ± 0.144**
Ni	**0.314 ± 1.29**	0.269 ± 0.688	0.0908 ± 0.058
Pb	0.0154 ± 0.008	0.0541 ± 0.071	**0.322 ± 0.972**
Se	0.304 ± 0.083	**1.52 ± 0.45**	1.37 ± 0.18
Sn	0.798 ± 0.085	2.78 ± 1.29	**3.29 ± 0.99**
V	0.0003 ± 0.0005	0.0015 ± 0.002	**0.0021 ± 0.0025**
Zn	3.00 ± 1.96	3.99 ± 4.59	**64.0 ± 13.0**

[a] Aluminum (Al), arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), vanadium (V), and zinc (Zn).
[b] ND = not detected.

(2016). Across three seabird species, Forster's Terns (*Sterna forsteri*), Black-necked Stilts (*Himantopus mexicanus*), and American Avocets (*Recurvisrostra americana*), metal content in eggs was highly correlated with concentrations in maternal blood, kidney, muscle, liver, and head feathers. Although such relationships have yet to be quantified for Humboldt Penguins, Ackerman et al. (2016) validate the relevance of drawing comparisons between eggs and tissues from adult birds across taxa.

Selenium is not a metal, but selenium concentrations were measured because Hg exhibits a high binding affinity for Se and acts to inhibit Se-dependent enzymes vital to metabolic pathways in the brain and neuroendocrine system (Ralston and Raymond 2010). As such, ratios exceeding 1:1 may indicate a protective effect of Se against Hg toxicity (Berry and Ralston 2008). Alternatively, a molar ratio ≤1 would be indicative of all available Se being bound to Hg, potentially resulting in oxidative stress risk if any unbound Hg existed (Cáceres-Saez et al. 2013). However, increased Se tissue concentrations (i.e., Se:Hg >1) preserves Se-dependent enzyme activity, subsequently mitigating Hg toxicity (Berry and Ralston 2008; Ralston et al. 2008; Ralston and Raymond 2010). As molar Se:Hg ratios exceeded 1:1 for all yolk and albumen samples, indicating that Hg present in these egg components was fully bound to Se, it is posited that the potential for oxidative stress due to mercury exposure during chick embryogenesis is reduced for Humboldt Penguins in our managed-care population.

Yolk

Of all egg components, yolk exhibited the highest concentrations for 10/16 elements; Mo was detected only in yolk and not in albumen or eggshell. The range of elements concentrated in yolk can be largely explained by the metabolomics of avian vitellogenesis. Lipid is the largest yolk constituent by dry mass (Nys and Guyot 2011). Certain metals, such as Mn and

TABLE 2. Kruskal-Wallis test outputs for aluminum (Al), arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), vanadium (V), and zinc (Zn) concentrations (μg/g) in egg components of Humboldt Penguins (*Spheniscus humboldti*) from a managed-care population at the Brookfield Zoo, Chicago, Illinois, USA. For comparisons where a significant difference in element content was found, the sample with higher concentration is denoted with boldface text.

Element[a]	Sample comparison	*H* value	*P* value	Significant difference?
Al	Eggshell + albumen	0.577	0.4474	No
	Eggshell + yolk	1.48	0.2242	No
	Yolk + albumen	0.475	0.4907	No
As	Eggshell + albumen	1.05	0.3051	No
	Eggshell + **yolk**	22.9	<0.0001	Yes
	Yolk + albumen	20.3	<0.0001	Yes
Cd	Eggshell + **albumen**	7.48	0.0062	Yes
	Eggshell + yolk	3.54	0.0601	No
	Yolk + albumen	0.762	0.3827	No
Co	Eggshell + **albumen**	3.9	0.0482	Yes
	Eggshell + **yolk**	20.1	<0.0001	Yes
	Yolk + albumen	14.5	0.0001	Yes
Cr	Eggshell + **albumen**	15.9	0.0007	Yes
	Eggshell + **yolk**	20.1	<0.0001	Yes
	Yolk + albumen	3.72	0.0536	No
Cu	Eggshell + **albumen**	22.2	<0.0001	Yes
	Eggshell + **yolk**	22.9	<0.0001	Yes
	Yolk + albumen	0.931	0.3346	No
Fe	Eggshell + albumen	2.55	0.1106	No
	Eggshell + **yolk**	22.9	<0.0001	Yes
	Yolk + albumen	20.3	<0.0001	Yes
Pb	Eggshell + **albumen**	6.67	0.0098	Yes
	Eggshell + yolk	1.67	0.1965	No
	Yolk + albumen	0.357	0.5503	No
Mn	**Eggshell** + albumen	13	0.0003	Yes
	Eggshell + **yolk**	22.9	<0.0001	Yes
	Yolk + albumen	20.3	<0.0001	Yes
Hg	Eggshell + **albumen**	22.9	<0.0001	Yes
	Eggshell + **yolk**	22.9	<0.0001	Yes
	Yolk + **albumen**	20.3	<0.0001	Yes
Mo	Eggshell + albumen	0.468	0.4941	No
	Eggshell + **yolk**	22.9	<0.0001	Yes
	Yolk + albumen	20.3	<0.0001	Yes
Ni	**Eggshell** + albumen	17.1	<0.0001	Yes
	Eggshell + yolk	17.1	<0.0001	Yes
	Yolk + albumen	0.076	0.7828	No
Se	Eggshell + **albumen**	22.9	<0.0001	Yes
	Eggshell + **yolk**	22.9	<0.0001	Yes
	Yolk + albumen	2.03	0.1543	No
Sn	Eggshell + **albumen**	16.8	<0.0001	Yes
	Eggshell + **yolk**	22.9	<0.0001	Yes
	Yolk + albumen	1.54	0.2140	No
V	Eggshell + **albumen**	9.01	0.0025	Yes
	Eggshell + **yolk**	12.8	0.0004	Yes
	Yolk + albumen	1.43	0.2322	No

TABLE 2. Continued.

Element[a]	Sample comparison	*H* value	*P* value	Significant difference?
Zn	Eggshell + albumen	0.175	0.6761	No
	Eggshell + **yolk**	22.9	<0.0001	Yes
	Yolk + albumen	20.3	<0.0001	Yes

[a] Aluminum (Al), arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), vanadium (V), and zinc (Zn).

Zn, are able to form lipophilic metal complexes that promote absorption and tissue penetration, increasing contaminant retention in yolk (Tjälve and Gottofrey 1991). These metals also tend to accumulate in organs responsible for toxin elimination, such as the liver (Das et al. 2003). The lipids vitellogenins and yolk-targeted very-low-density lipoproteins (VLDLy) are synthesized in the liver, secreted into the circulatory system, and taken up within the ovary's follicles for generation of yolk, the nutrient source for the developing embryo (Walzem 1996; Vezina et al. 2003). Once transported to the oocyte, vitellogenin is catabolized to phosvitin and lipovitellin, the predominant proteins found in yolk (Hermier et al. 1989; Speake et al 1998). Phosvitin exhibits a high capacity to bind essential metals (e.g., Ca and Fe), hence their enrichment in yolk (Strixner and Kulozik 2011).

During yolk development, ~17 d in Humboldt Penguins (Ancel et al. 2013), hepatic lipid production increases as lipoprotein synthesis shifts to generate the smaller, lipid-rich VLDLy molecules required to support an embryo. As plasma vitellogenin concentrations increase, a corresponding rise in trace mineral concentrations occurs due to vitellogenin-metal binding (Hill 1974; Panic et al. 1974; Richards 1989). Increases in VLDLy and vitellogenin production by the liver necessitate a corresponding increase in hepatocyte metabolism (Vezina et al. 2003). Richards (1997) attributes yolk heavy metal content almost entirely to vitellogenesis-driven increases in hepatic metabolism, which in turn facilitate maternal transfer of contaminants to the developing egg via the circulatory system.

The trends in yolk metal accumulation that we found have been seen in other birds, as vitellogenesis mechanisms are conserved across avian species. Duck eggs from commercial farms in Thailand had yolk concentrations of Fe, Mn, and Zn an order of magnitude greater than those in albumen (Aendo et al. 2018), a trend reflected in our Humboldt Penguin eggs (Table 3). Humboldt Penguin yolk Cu, Hg, and Pb concentrations (Table 1) exceeded wet weight concentrations in Taiwanese duck eggs (Jeng and Yang 1994) by an order of magnitude of 19. However, concentrations in commercial chicken eggs (Demirulus 2013; Sarkar et al. 2018), were consistently higher than those we observed in Humboldt Penguin eggs; this might be attributable to herbicide or pesticide contamination of feed residues (Sarker et al. 2017; Table 3). Yolk Pb concentrations (0.322 μg/g; Table 1) in our study were similar to those found in the homogenized internal egg components of Great Cormorants (*Phalacrocorax carbo*) in Greece (Goutner et al. 2001). Although values for yolk of Pied Avocets (*Recurvirostra avosetta*), Yellow-legged Gulls (*Larus cachinnans michahellis*), and Mediterranean Gulls (*Larus melanocephalus*) from the Evros and Axios deltas in Greece were an order of magnitude greater than those penguin yolk, these birds also occupy habitats with considerable pollutant input from freshwater discharge generated by local agricultural complexes and commercial aquaculture ponds (Goutner et al. 2001). Compared to homogenized egg contents collected from Yellow-breasted Chats (*Icteria virens*) and Willow Flycatchers (*Empidonax traillii*) from Arizona, USA (Mora 2003), Humboldt Penguin yolk values exceeded wet weight concentrations of Cu, Mn, and Zn but were lower in Se (Table 3). This may be attributable to the managed-care Humboldt Penguins occupying a higher trophic niche than either shorebird species, with contaminant biomagnification subsequently resulting in greater metal accumulation in the penguins' egg components.

Mean As (0.20 μg/g), Mn (2.71 μg/g), Pb (0.322 μg/g), and Zn (64.03 μg/g; Table 1) concentrations in Humboldt Penguin yolk fell within the range of mean concentrations reported for feathers from the Antarctic penguins Adelie Penguins (*Pygoscelis adeliae*; Dos Santos et al. 2006), Chinstrap Penguins (*Pygoscelis antarctica*; Jerez et al. 2013b), and Gentoo Penguins (*Pygoscelis papua*; Metcheva et al. 2006; Jerez et al. 2011, 2013a;). Although As, Mn, and Zn concentrations in kidney, liver, and muscle of these species exceed managed-care Humboldt Penguin yolk concentrations by up to an order of magnitude (Metcheva et al. 2010; Jerez et al. 2013a, b), the same tissues are significantly lower than yolk for Pb. Yolk Pb values were also similar to those in feathers of Magellanic Penguins (*Spheniscus magellanicus*) from the coast of southern Brazil (Kehrig et al. 2015), and yolk As and Zn concentrations were similar to those in feathers of Little Penguins (*Eudyptula minor*) from three island rookeries near Melbourne, Australia (Finger et al. 2015).

Yolk was an order of magnitude higher in Hg and two orders of magnitude higher in Zn than serum from wild Humboldt Penguins at PSJ but comparable in Al concentrations. Wild Humboldt Penguin feathers at PSJ had significantly higher concentrations of Al, As, Cu, Fe, Hg, and Mn than yolk from the eggs that we tested (Adkesson 2019). Humboldt Penguin excreta from three Chilean rookeries had mean As and Pb concentrations 40 times greater and mean Zn eight times higher (Celis et al. 2014) than in the yolks we tested (Table 4). Heightened metal concentrations in wild birds are to be expected because of the prevalence of mines and generalized anthropogenic activity throughout coastal Peru and Chile (Adkesson 2019).

Data for other heavy metals that we found to be highly concentrated in the yolk (Co [0.01 μg/g], Cr [0.086 μg/g], Mo [0.57 μg/g], and Sn [3.29 μg/g]; Table 1) are not well established in the literature for penguins.

Albumen

We found the highest concentrations of Al, Cd, Cu, Hg, and Se in albumen, a lipid-free fluid comprising primarily water and proteins, as well as vitamins and minerals required for embryonic development (Strixner and Kulozik 2011). Unlike yolk constituents, which are derived from components synthesized remotely (i.e., in the liver), albumen proteins are synthesized and secreted locally by the magnum of the oviduct (Nys and Guyot 2011). Ovalbumin and ovotransferrin, the two principal albumen proteins, form metal complexes that offer protection from bacterial infections (Schiavone and Barroeta 2011). Ovalbumin binds both Se and Hg (Magat and Sell 1979; Latshaw and Biggert 1981), as well as Cu, Mn, and Zn (Goux and Venkatasubramanian 1986). Ovotransferrin is principally responsible for Fe binding (Schiavone and Barroeta 2011), but Al, Cd, Cr, Co, Cu, Mn, and Zn bind in vitro (Burley and Vadehra 1989). The highly positive correlation between Mn and Fe in Humboldt Penguin albumen may result from Mn interacting with Fe cofactors on enzymes and impinging on transferrin receptor expression, limiting Fe endocytosis into red blood cell precursors, hepatocytes, and endothelial cells (Gupta et al. 2007).

Mercury levels in birds are well established due to the toxicity associated with its bioaccumulation and biomagnification. Humboldt Penguin mean Hg concentrations in albumen (0.62 μg/g; Table 1) were five times higher than levels found in wild Gentoo Penguin albumen (Brasso et al. 2012). This likely results from managed-care birds receiving higher trophic prey items than those foraged in the wild, because Hg levels in albumen from eggs laid by managed-care Gentoo Penguins are higher than albumen from wild birds (Brasso et al. 2012).

Notably, Hg in Humboldt Penguin albumen (0.62 μg/g; Table 1) far exceeded values in wild Magellanic Penguin feathers (0.033 μg/g; Frias et al. 2012) despite the species' nearly identical trophic dynamics and ecological niche (Simeone et al. 2009). Additionally, Humboldt Penguin albumen is comparable to values found in

TABLE 3. Mean metal concentrations (µg/g) in the egg components of various bird species compared to those in egg components of Humboldt Penguins (*Spheniscus humboldti*) from a managed-care population at the Brookfield Zoo, Chicago, Illinois, USA.

Species	Sample type	Al[a]	As[a]	Cd[a]	Cu[a]	Hg[a]	Mn[a]	Pb[a]	Zn[a]	Literature source
Domestic Duck (*Anas platyrhynchos domesticus*)	Albumen	—[b]	—	0.18	3.38	—	0.10	2.21	1.66	Aendo et al. (2018)
	Yolk	—	—	0.14	1.79	—	1.37	1.85	50.05	
	Albumen	—	—	0.0018	0.83	0.0178	—	0.0136	—	Jeng and Yang (1995)
	Yolk	—	—	0.0038	0.00136	0.0097	—	0.0847	—	
Chicken (*Gallus gallus domesticus*)	Albumen	—	—	0.78	4.2	—	3.2	—	5.8	Demirulus (2013)
	Yolk	—	—	0.79	6.6	—	4.3	—	38.9	
	Albumen	—	0.160	0.039	0.899	—	—	0.106	—	Sarkar et al (2018)
	Yolk	—	0.157	0.042	0.882	—	—	0.123	—	
Willow Flycatcher (*Empidonax traillii*)	Homogenized internal components	—	<0.5	—	2.5	—	1.9	<0.5	38.5	Mora (2003)
Yellow-breasted Chat (*Icteria virens*)	Homogenized internal components	—	<0.5	—	3.2	—	2.7	<0.5	52.4	
Great Cormorant (*Phalacrocorax carbo*)	Homogenized internal components	—	—	—	0.003	—	—	0.008	—	Goutner et al. (2001)
Pied Avocet (*Recurvirostra avosetta*)	Homogenized internal components	—	—	—	0.006	—	—	0.07	—	
Yellow-legged Gull (*Larus cachinnans michahellis*)	Homogenized internal components	—	—	—	0.005	—	—	0.068	—	
Mediterranean Gull (*Larus melanocephalus*)	Homogenized internal components	—	—	—	0.003	—	—	0.020	—	
Little Egret (*Egretta garzetta*)	Homogenized internal components	—	0.224	0.001	6.583	0.137	1.493	0.014	51.38	Lam et al. (2005)
	Shell	—	—	0.006	1.602	0.071	4.164	0.152	9.636	
Black-crowned Night Heron (*Nycticorax nyycticorax*)	Homogenized internal components	—	—	—	6.286	0.520	1.720	0.007	39.87	
	Shell	—	—	0.008	1.116	0.056	1.031	0.030	5.390	
Bridled Tern (*Sterna anaethetus*)	Homogenized internal components	—	1.38	0.002	3.920	0.562	2.636	0.010	47.62	
	Shell	—	0.397	0.002	1.235	0.004	1.112	0.060	2.351	

TABLE 3. Continued.

Species	Sample type	Al[a]	As[a]	Cd[a]	Cu[a]	Hg[a]	Mn[a]	Pb[a]	Zn[a]	Literature source
Humboldt Penguin (*Spheniscus humboldti*)	Shell	—	3.61	0.0007	1.32	—	0.498	0.0154	3.0	Present study
	Albumen	0.0164	4.19	0.0009	4.02	0.618	0.171	0.0541	3.99	
	Yolk	0.195	3.86	0.0006	3.77	0.079	2.71	0.322	64	

[a] Arsenic (As), aluminum (Al), cadmium (Cd), copper (Cu), mercury (Hg), lead (Pb), and zinc (Zn).
[b] — = not tested for this element.

Adelie (Carravieri et al. 2016) and Gentoo Penguin feathers (Dos Santos et al. 2006; Table 4).

Concentrations of Cu (4.02 μg/g), Hg (0.618 μg/g), and Pb (0.0541 μg/g) in Humboldt Penguin albumen were greater than those found in Thai (3.38 μg/g for Cu, 2.21 μg/g for Pb; Aendo et al. 2018), and Taiwanese duck eggs (0.00136 μg/g, 0.0178 μg/g, and 0.0146 μg/g, respectively; Jeng and Yang 1994). Additionally, penguin albumen had higher Cd levels than yolk, a trend also observed in ducks (Aendo et al. 2018) that is likely attributable to ovotransferrin binding (Burley and Vadehra 1989). However, much like for yolk, heavy metal concentrations in commercial chicken egg albumen have been found to be significantly greater than those in Humboldt Penguin egg albumen (Demirulus 2013; Sarkar et al 2018; Table 3).

The mean albumen Al (4.19 μg/g) that we found resembled concentrations in the kidneys and livers of Adelie, Chinstrap, and Gentoo Penguins (Jerez et al. 2013a), while mean Cd levels (0.0009 μg/g) were lower than values in these penguins across tissue types (Jerez et al. 2013a; Table 4). While mean Al concentrations in feathers from Humboldt Penguin at PSJ far exceeded mean egg albumen levels in the managed-care population, serum Al levels in the wild birds were approximately half that seen in albumen (Adkesson et al. 2019). Albumen also contained up to two orders of magnitude higher Hg and Zn than serum collected from wild Humboldt Penguins at PSJ. Concentrations of Se (1.52 μg/g) were highest in albumen; however, much like Co, Cr, Mo, Sn, and V in yolk, Se concentrations are not reported for penguins (Table 4).

Eggshell

Eggshell had the lowest concentration of all analyzed metals except Ni (0.31 μg/g). This was unexpected, as certain divalent metals, specifically Cd and Pb, mimic and replace Ca during shell biomineralization (Strixner and Kulozik 2011). The replacement of calcium by its mimics results in the inhibition of Ca precipitation in calcified tissues (Wada et al. 1995; Bridges and Zalups 2005), resulting in metal increases in eggshells (O’Flaherty 1991;

TABLE 4. Mean metal concentrations (μg/g) in the eggs and tissues of various penguin species compared to those in egg components of Humboldt Penguins (*Spheniscus humboldti*) from a managed-care population at the Brookfield Zoo, Chicago, Illinois, USA. For comparisons where an element was not tested, a dash is used in lieu of a concentration value.

Region	Species	Sample type	Al	As	Cd	Cu	Hg	Mn	Pb	Zn	Literature source
Antarctica	Adelie Penguin (*Pygoscelis adeliae*)	Feather	43.4	0.17	0.13	19.3	0.66	2.01	0.32	83.9	Jerez et al. (2013a, b); Carravieri et al. (2016)
		Kidney	4.09	0.44	0.2	14.8	—	11.18	0.05	234	Jerez et al. (2013a)
		Bone	—	0.13	0.01	0.96	—	8.31	0.04	227	
		Liver	6.81	0.6	0.06	92.1	—	12.01	0.04	133	
		Muscle	—	—	0.01	6.4	—	1.5	0.12	104	Smichowski et al. (2006)
	Chinstrap Penguin (*Pygoscelis antarctica*)	Feather	26	0.01	0.1	20.3	—	2.25	1.73	97.3	Jerez et al. (2011)
		Kidney	10.93	0.5	0.54	13.6	—	10.19	0.14	92.8	Jerez et al. (2013b)
		Bone	—	0.08	<0.001	0.71	—	12.5	0.14	235	Jerez et al. (2013a)
		Liver	15.52	0.47	0.11	132	—	11.42	0.18	132	Jerez et al. (2013b)
		Muscle	—	—	0.01	6.82	—	2.55	0.2	105	
	Gentoo Penguin (*Pygoscelis papua*)	Feather	46	0.07	0.21	17	0.54	2.6	0.1	106	Dos Santos et al. (2006); Metcheva et al. (2006); Jerez et al. (2011)
		Kidney	6.91	0.4	0.2	14.3	—	7.54	<0.001	125	Jerez et al. (2013a)
		Bone	—	0.19	0.001	1.15	—	11.01	<0.001	245	Barbosa et al. (2013)
		Liver	2.12	0.45	0.08	142	—	10.51	<0.001	52.9	Jerez et al. (2013a)
		Muscle	—	—	0.01	—	—	1.46	<0.001	106	
		Eggshell	28.9	—	<0.05	1.24	0.05	0.82	0.68; 0.75	4.07	Yin et al. (2008); Metcheva et al. (2011)
	King Penguin (*Aptenodytes patagonicus*)	Feather	—	—	—	—	1.98	—	—	—	Scheifler et al. (2005)
	Northern Rockhopper Penguin (*Eudyptes moseleyi*)	Feather	—	—	—	—	2.1	—	—	—	Carravieri et al. (2016)
	Southern Rockhopper Penguin (*Eudyptes chrysocome*)	Feather	—	—	—	—	1.96	—	—	—	Carravieri et al. (2013)
	Macaroni Penguin (*Eudyptes chrysolophus*)	Feather	—	—	—	—	2.24	—	—	—	

TABLE 4. Continued.

Region	Species	Sample type	Al	As	Cd	Cu	Hg	Mn	Pb	Zn	Literature source
Australia	Little Penguin (*Eudyptula minor*)	Feather	40.4	0.18	0.06	—	4.13	—	0.42	84.7	Finger et al. (2015)
South America	Magellanic Penguin (*Spheniscus magellanicus*)	Feather	—	—	—	—	0.033	—	0.14	—	Frias et al. (2012)
		Kidney	—	—	—	—	—	—	0.55	—	
		Liver	—	—	7.25	—	—	—	5.7	—	
	Humboldt Penguin (*Spheniscus humboldti*)	Excreta	—	7.86	—	—	—	—	12.79	487	Celis et al. (2014)
		Serum	2.14	—	—	—	0.0024	—	—	0.57	Adkesson et al. (2019)
		Feather	67	5.0	—	10.5	1.21	5.7	—	48	
		Shell	3.61	—	0.0007	1.32	—	0.498	0.0154	3.00	Present study
		Albumen	4.19	0.0164	0.0009	4.02	0.618	0.171	0.0541	3.99	
		Yolk	3.86	0.195	0.0006	3.77	0.079	2.71	0.322	64	

[a] Aluminum (Al), arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), selenium (Se), tin (Sn), vanadium (V), and zinc (Zn).

Teodorova et al. 2003; Scheuhammer 1996). Accordingly, Cd and Pb are expected to concentrate in the eggshell but were found in greater concentrations in albumen and yolk.

Humboldt Penguin heavy metal concentrations in eggshell were comparable to Gentoo Penguin eggshells for Al, Cu, Mn and Zn; Gentoo Penguin eggshell had higher metal concentrations (Metcheva et al. 2011). Likewise, all analyzed metals were found in higher concentrations in the feathers and serum of wild penguins at PSJ than in our Humboldt Penguin eggs (Adkesson et al. 2019; Table 4).

However, our Humboldt Penguin eggshells were higher in As (3.61 µg/g) than eggshells collected from Bridled Terns (*Sterna anaethetus*; 0.397 µg/g) inhabiting the Pearl River Delta manufacturing complex near Hong Kong (Lam et al. 2005). Eggshells of Little Egrets (*Egretta garcetta*) and Black-crowned Night Herons (*Nycticorax nycticorax*) inhabiting the same industrial complex had higher quantities of Cd, Mn, and Pb, while Zn concentrations were within the same range (2.351 µg/g to 9.636 µg/g) as our Humboldt Penguin eggshells (3.00 µg/g; Table 3). Managed-care birds represent a population with a significantly reduced risk of environmental contaminant exposure, yet the penguins assessed in this study nevertheless had accumulated metals in their eggshells to an extent comparable to findings in the wild birds assessed by Lam et al. (2005). Thus, when considering Humboldt Penguin populations inhabiting rookeries near coastal mines in Peru and Chile, we conjecture that there is an immense potential for significant contaminant accumulation in wild penguin eggshells, because of the introduction of metals and other environmental contaminants from local ore generation.

Implications for wild Humboldt Penguins

A recent ecosystem health assessment of Peruvian marine protected areas (MPAs), Loaiza et al. (2022) designated Punta San Juan, home to the largest Peruvian Humboldt Penguin rookery, as having the highest incidence of adverse biological effects and highest metal concentrations for As, Cd, Cu, Ni, Pb, and Zn in sediment and seston (particles suspended in water) out of all surveyed MPAs, because of its proximity to mines. Heavy metal exposure during embryonic development has been heavily implicated in teratogenesis and developmental deformities in birds (Gilani and Alibhai 1990). Accordingly, the potential for reductions in offspring viability due to heavy metal contamination in eggs is a concern for species exhibiting multidecadal population declines, such as the Humboldt Penguins. Our findings serve to substantiate the value of assessing eggs when conducting assessments of environmental contamination in these birds.

It is important to note that the penguins in this study remained in a controlled environment (i.e., consistent diet and environment) throughout oogenesis and egg laying. Variance in habitat, diet, and environmental exposure risk experienced by wild birds significantly influences their assimilation of inorganic contaminants, and this must be taken into consideration. Additionally, most published studies assessing heavy metal contamination in eggs have presented data in terms of eggshell and homogenized internal components (i.e., combined yolk and albumen), limiting the extent to which some comparisons could be made. In the future, quantification of heavy metal concentrations in wild Humboldt Penguin egg components should be pursued to fully assess concerns regarding heavy metal exposure and contamination to the chick embryo. By determining the extent of heavy metal offloading that occurs via egg production, linkages among contaminants, embryo viability, and the likelihood of post-hatch chick mortality may be established. Such data would significantly inform existing strategies for the management of wild Humboldt Penguins, facilitating the conservation of this species.

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