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Trace element enrichment in the eggshells of *Grus japonensis* and its association with eggshell thinning in Zhalong Wetland (Northeastern China)

Jinming Luo[1], Yajie Ye[2], Zhongyan Gao[3] & Wenfeng Wang[3]

[1] *Department of Science, Qiqihar University, Cultural St 42#, 161006, Qiqihar, P. R. China; email: Luojm1000@sina.com*
[2] *Department of Chemical Engineering, Qiqihar University, Cultural St 42#, 161006, Qiqihar, P. R. China*
[3] *Zhalong National Natural Reserve, Qinyun St. 221#, 161003, Qiqihar, P. R. China*

Abstract: The concentrations of six elements (Ca, Mg, Zn, Cu, Pb and Cd) in the eggshells of breeding red-crowned cranes (*Grus japonensis*) in China's Zhalong Wetland were examined. Two macronutrients, namely, Ca and Mg, were predominant in the eggshells, with corresponding concentrations varying from 127 g kg^{-1} to 323 g kg^{-1} and 0.33 g kg^{-1} to 5.51 g kg^{-1} (dry weight, dw), respectively. The Cu and Zn contents were in the range of below the detection limit (ND) to 8.05 mg kg^{-1} and ND to 511 mg kg^{-1} dw, respectively. The maximal Pb and Cd concentrations in the eggshells of the red-crowned crane exceeded a level considered to be potentially toxic in birds (i.e., 1.7 mg kg^{-1} for Pb and 0.34–0.91 mg kg^{-1} dw for Cd), ranging from ND–3.20 mg kg^{-1} dw for Pb and ND–1.20 mg kg^{-1} dw for Cd. The principal components analysis results revealed the complex chemical associations between two essential (Cu and Zn) and nonessential (Pb and Cd) elements in the eggshells of *G. japonensis* in China. Increased Pb and Cd levels in the bodies and eggshells of the red-crowned cranes suggest this species in their colonies are exposed to these contaminants, although which had not induced to obvious eggshell thinning in the examined species.

Key words: cadmium enrichment; residual eggshell; red-crowned crane; chemical association

Introduction

The excessive intake of trace elements has attracted increasing attention because of the toxic effects of these elements on the environment and the whole biota (Ebrahimpour 2008; Luo et al. 2013). Eggs of female oviparous organism are one of crucial ecological receptors for the toxic elements from their bodies (Dauwe et al. 1999). Thus, avian eggshells are frequently used to indicate environmental health of contaminated areas (Burger 1994; Dauwe et al. 1999; Hashmi et al. 2013). Macronutrients calcium (Ca) and magnesium (Mg) are basic components of eggshells. Dietary Ca deficiency may result in the accumulation of hazardous elements, such as Pb and Cd, in the eggshells (Scheuhammer 1996), thereby, changing the thickness and microstructure of eggshells (Gonzalez et al. 1988; Dauwe et al. 2006; Orlowski et al. 2014). Previous studies have shown that avian eggshell thinning caused by high dosage of organochlorine pesticides (OCPs) and trace elements (e.g., like Pb, Cd and Hg) would make the egg breakable, reducing the survival of the embryos and the reproduction rate (Miljeteig et al. 2012; Orlowski et al, 2014). Thus, eggshell thinning induced by the contaminant is a major threat to avian population. Consequently, determining the trace element concentrations in the eggshells and chemical interactions among trace elements is important to understand the ecological health of species at the top trophic levels in an aquatic system.

The red-crowned crane, *Grus japonensis* (Müller, 1776) is a rare species that has been near extinction since 2000, as indicated in the Red List of Endangered Species (BirdLife International 2012). Its global population is small at 2,750 mature individuals. Although the resident population in Japan remains stable (Teraoka et al. 2007), the migratory population in mainland Asia continuously dwindles because of the loss and degradation of wetlands for agricultural and industrial development (Cao & Liu 2008). Excessive levels of metals in the bodies and eggs of these avian could also have an adverse effect on their ecological health and contribute to their declining population.

We recently reported relatively high Pb and Cd concentrations in the sediment and prey of red-crowned crane in northeastern China (Luo et al. 2013, 2015c). Relatively high Pb (2.1–5.8 mg kg^{-1}) and Cd (1.42–3.06 mg kg^{-1}) contents have been detected in the molten feathers and bodies of some red-crowned cranes (Luo et al. 2015b, c). However, whether Cd-rich prey contributes to nutrient (e.g., Ca and Mg) deficiency and eggshell thinning remained unanswered. This study was performed to monitor the concentrations of Ca, Mg, Cu, Zn, Pb and Cd, in association with eggshell thinning of

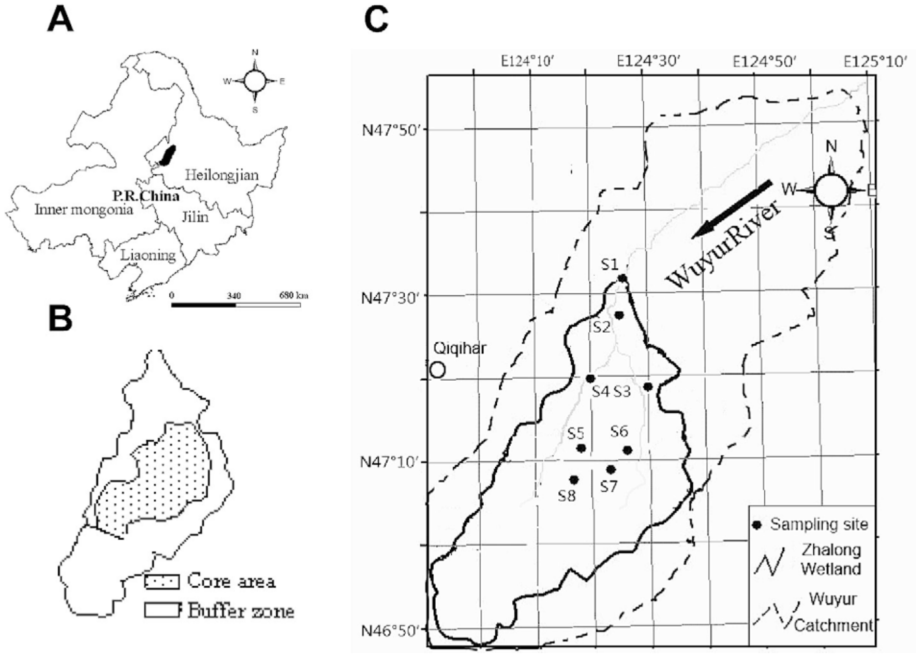

Fig. 1. Location of Wuyur catchments (A, B) and sampling sites of residual eggshells left by red-crowned crane (C).

the red-crowned cranes in Zhalong Wetland in China. Here we hypothesized that rich-Cd prey had brought about effect on the thickness of the eggshells and contributed to population decline in the examined species. Our findings will help improve the current understanding on the tissue distribution of the macro and trace elements, especially contaminants, in the eggshells of red-crowned crane.

Material and methods

Study site

Wuyur River originates from the western foot of Xiaoxin'an Mountain, Northeastern China, where the watershed is an elongated strip that flows through main food production zone of Heilongjian province in China (Figs 1A, B). The lower reaches of the river disappear after entering into the Zhalong Wetland, and develop a large area of reed marsh. Zhalong Wetland covers an area of 2,100 km^2 (123°51′–124°37′ E, 46°48′–47°32′ N). The core area is approximately 700 km^2, with the buffer zone occupying 1,400 km^2 lying outside of the core area (Fig. 1C). Large areas of pristine reed marsh in the wetland attract more than 500 migratory red-crowned cranes to inhabit and breed from late March to early November (approximately eight months) every year.

In recent years, various sludge and wasted water from the surrounding residential area, agricultural land, and industrial workshop containing several types of toxic contaminants, including Zn, Cd, and other trace elements, are discharged directly into the wetland without complete disposal treatment. The wastewater discharge volume increased from 0.17×10^8 m^3 in 1993 to 0.45×10^8 m^3 in 2010. Increasing amount of pollution discharges has elevated the concentrations of various trace elements in the sediment and aquatic biota (Luo et al. 2015c).

Sampling scheme

Surface sediment samples from 35 sampling sites in the wetland were collected using a sediment grab sampler. The samples were immediately packed in dark-colored polyethylene bags, refrigerated, and then transported back to the laboratory. Six typical aquatic animal taxa, including three invertebrates [water beetle, *Cybister japonicus* Sharp, 1973 (Dytiscidae), pond snail, *Cipangopaludina chinensis* (Gray, 1834) (Viviparidae), and dragonfly, *Aeshna mixta* Latreille, 1805 (Odonata)] as well as three fish species with body size smaller than 10 cm [common carp, *Cyprinus carpio* L., 1758 (Cyprinidae), pond loach, *Misgurnus mohoity* (Dybowski, 1869) (Cobitidae), and Chinese sleeper, *Perccottus glenii* Dybowski, 1877 (Odontobutidae)], that are typical prey of wild red-crowned crane in the wetland, were collected in three regions (buffer zone and core area) (see details in Luo et al. 2015c). All prey samples were rinsed thoroughly in the field with distilled water to remove pollutants attached to their body. The samples were placed in a car refrigerator at –4 °C, and transported back to the laboratory.

All sediment samples were sieved through a 63 μm mesh after indoor air drying. They were then digested with acid, based on the method by Viklander (1998). The aquatic animals were first dried with filter paper, and then oven-dried to constant (48 h at 60 °C). The dried samples were ground to homogeneous powders in a quartz bowl for acid digestion.

Red-crowned crane carcasses collection process was employed in the field from 2010 to 2014. A total of eight red-crowned crane carcasses were collected at 8 nesting sites (Fig. 1C, Table 1), including five males and three females, aided by the fire inspectors of Zhalong Wetland. According to a pathological inspection, the direct cause of death of these crane samples were starvation because of food shortage in freezing condition, or collision on the power line (Luo et al. 2015a).

Hatched residual eggshells were collected from the field in late April and early May of 2004, 2010 and 2014. A total of 24 residual eggshells left by red-crowned cranes were collected at the nesting sites S1–S8. The eggshells were washed with distilled water in the field to remove the inner membrane and other contaminants from sediment and immediately transferred to laboratory. The thickness of the eggshell

Table 1. Habitat, presumed cause of death, and body weight (B.W.) of eight carcasses of red-crowned cranes.

Site	Habitat	Cause of possible death	Finding date	Sex	Adult/Juvenile	B.W. (kg)
S1	Reed marsh	Collision with power lines	Apr 2014	Male	Adult	9.8
S2	Reed marsh	Collision with power lines	Apr 2014	Male	Juvenile	6.2
S3	Reed marsh	Accidental injury	Apr 2010	Male	Adult	9.4
S4	Meadow	Accidental injury	Oct 2010	Male	Juvenile	6.8
S5	Meadow	Starvation, accidental injury	Nov 2012	Female	Juvenile	6.5
S6	Reed marsh	Starvation, accidental injury	May 2011	Female	Adult	6.5
S7	Meadow	Accidental injury	Apr 2014	Female	Juvenile	5.8
S8	Meadow	Starvation, accidental injury	Oct 2012	Male	Adult	8.3

Table 2. Heavy metals concentrations in surface sediments of Zhalong Wetland (mg kg^{-1} in dw; $n = 34$).

		Cu	Zn	Pb	Cd
Buffer zone	Mean ± SD	43.87 ± 19.88	69.22 ± 40.35	81.49 ± 33.44	4.70 ± 1.37^{ab}
	Range	(16.10–88.33)	(53.64–125.58)	(90.70–125.58^{ab})	(2.39–6.14^{ab})
	EF	(0.78–4.27)	(0.77–1.80)	(4.03–5.58)	(15.93–40.93)
Core area	Mean ± SD	20.75± 8.82	59.35± 35.45	55.11± 23.84	2.83± 0.99
	Range	(5.29–37.21)	(25.58–137.35)	(9.85–79.08)	(1.23–4.65^{ab})
	EF	(0.26–1.80)	(0.37–1.97)	(0.44–3.52)	(8.20–31)
Background levels		20.7	69.61	22.49	0.15
Tolerable level		100	300	100	3

Explanations: [a]Concentration exceeded probable effect level values (PELs), as reported by McDonald et al. (2000); [b]Tolerable level for agro-economic crops, as reported in Kabata-Pendias (2001); EF (enrichment factor) = observed concentration/background levels; Background levels were reported by Li & Zheng (1988).

was measured by micrometer (ShenZhen Sun-Time Scientific Co. Ltd, China) with an accuracy of 1 μm in the laboratory. After drying with filter papers, the eggshells were oven-dried to constant weight (48 h at 60 ℃).

Microwave digestion and element analysis

A total of 0.5 g of sediment from each site was acid-digested in a microwave according to USEPA (1996) methods. Approximately 1 g to 2 g samples of internal tissues (i.e., liver, kidney, and gut wall) and 0.5 g eggshell were collected for acid-digestion. Polyethylene gloves were used throughout the all dissection procedures to prevent contamination. Both internal tissues and eggshells were acid digested in a microwave oven (MDS-15, Sineo Microwave Chemistry Technology Col, LTD, Shanghai of China) were digested according to the methods by Canli, Ay, and Kalay (1998). Triplicate sub-samples of known dry weight were digested in acid mixture (3 ml HNO_3 + 1 ml HCl; both chemicals were analytical reagent and supplied from Shenzhen three chemical co., LTD of China), evaporated slowly to almost dryness (90 ℃), and the residue was dissolved in 5 ml 1 : 1 diluted HCl, and then settled to 25 ml for analysis after the solution cooled down to room temperature.

The determination of Cu, Zn, Ca, Mg, Pb, and Cd concentrations in each sample category, i.e., sediment, aquatic animal, the internal tissues (liver, kidney, and gut wall) and external tissues eggshell of the red-crowned crane, was determined using inductively coupled plasma-mass spectrometry (ICP-MS Agilent 7500ce, Agilent Technologies, Inc. of U.S. America). The detection limits of Ca and Mg were 0.5 mg kg^{-1}, and 0.05 mg kg^{-1} for the other elements. We estimated the precision and accuracy of the analyses based on a certified reference materials (Beijing Shiji Ouke Biotech Co., Ltd of China): *Pseudoscianea crocea* (GBW08573) for Cu (1.36 ± 0.13 mg kg^{-1}), Zn (28.8 ± 1.4 mg kg^{-1}), Pb (8.8 ± 1.10 mg kg^{-1}), and Cd (0.014 ± 0.001 mg kg^{-1}) with the measured values (trace metals in eggshell of the red-crowned crane). The results agreed with the certified values for all metals, with average recovery rates of 102% for Cu, 94% for Zn, 103% for Pb, and 105% for Cd. All materials used for sampling and analysis were acid-washed. Moreover, all samples were analyzed in triplicate at a relative standard deviation lower than 1.5%.

Statistical analysis

The correlation coefficients of eggshell thickness and metal concentrations were computed based on the average metal concentrations in the eggshells and average eggshell thickness. Before statistical analyses, logarithmic transformation of the data was performed in case they did not meet the assumption of normality distribution. SPSS 10.0 for Windows was used for data analysis.

Analysis of variance (ANOVA) was employed to test whether the metal concentrations in the sediment varied significantly in different ecological zones (Table 2) and whether the thickness of the eggshell got thinning in recent years (Fig. 3). A *post-hoc* comparison (Tukey test) was used as a follow-up test to ANOVA to show the statistical differences between areas. Values at $P < 0.05$ were considered statistically significant. Pearson's correlation coefficients were used to calculate correlations of the selected six trace elements in the eggshell of red-crowned crane (Table 5), as well as eggshell thickness versus the toxic element concentration. A PCA varimax-normalized factor rotation procedure was applied to identify the potential mutual associations of individual elements. Factor loadings were used to interpret the complicated chemical associations.

Results

Trace elements in the prey

The average Cu and Zn concentrations in the sediments were slightly higher than natural background values in the region (Table 2). Two non-essential elements, Pb

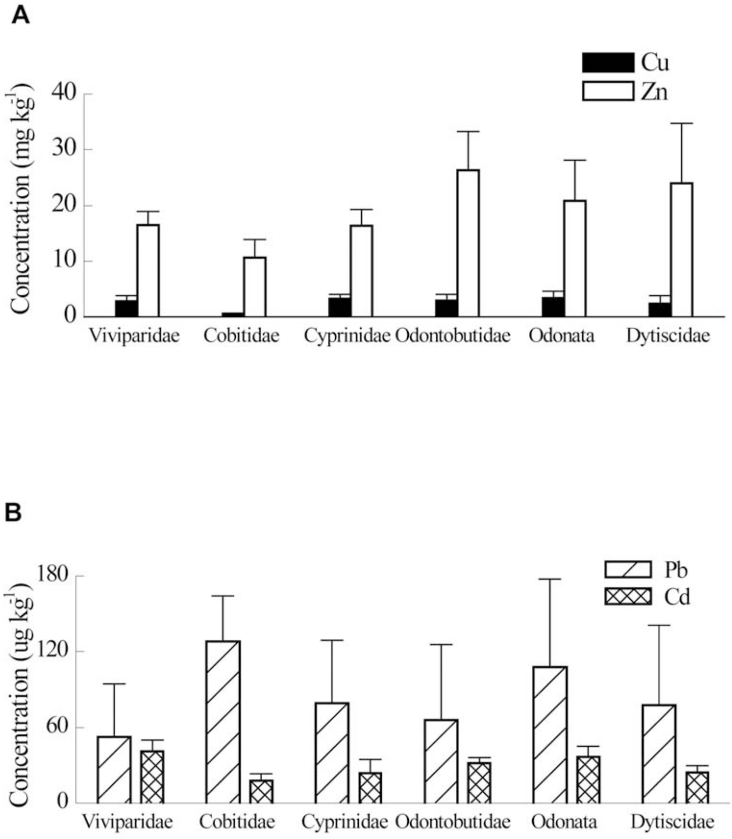

Fig. 2. Concentrations (Means + SD) of two essential metals (A) and three nonessential metals (B) in six aquatic animal taxa. Note units difference in Fig. 2A and Fig. 2B: mg kg^{-1} for milligrams per kg and μg kg^{-1} for micrograms per kg.

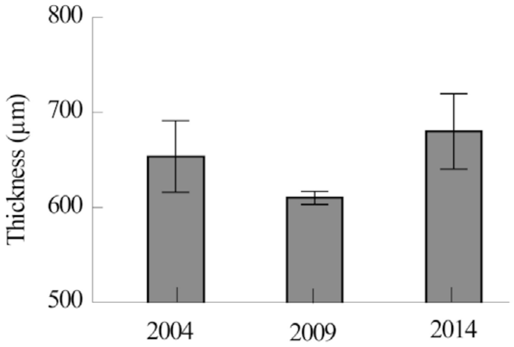

Fig. 3. Change of eggshells thickness of red-crowned crane in Zhalong Wetland in 2004, 2009 and 2014.

and Cd, were quantified in the sediments, and the average concentrations were higher than the background levels (22.49 mg kg^{-1} for Pb and 0.15 mg kg^{-1} for Cd), i.e., 90.70 mg kg^{-1}and 4.70 mg kg^{-1} in the buffer zone and 55.11 mg kg^{-1} and 2.83 mg kg^{-1} in the core area.

Detectable concentrations of four trace elements were observed in the aquatic animals (the prey of red-crowned crane) in the following order: Zn > Cu > Pb > Cd (Fig. 2). Zn and Cu were dominant in the aquatic animal families, with average concentrations ranging from 11.04 mg kg^{-1} to 39.48 mg kg^{-1} and 0.76 mg kg^{-1} to 4.66 mg kg^{-1}, respectively. Relatively high Pb and Cd concentrations were found in the six groups of aquatic animals, with corresponding average concentrations ranging from 8.37 μg kg^{-1} to 48.84 μg kg^{-1} and 13.45 μg kg^{-1} to 53.26 μg kg^{-1}.

Enrichment of trace element in the internal tissues and eggshells

The element concentrations varied greatly and in the following order: Ca > Mg > Zn > Cu > Pb > Cd (Table 3). The dominant macro-elements Ca and Mg in the eggshell varied from 127 g kg^{-1} to 323 g kg^{-1} and 0.33 g kg^{-1} to 4.18 g kg^{-1} (dw), respectively. The corresponding Cu and Zn were quantified in the eggshells of the red-crowned cranes ranged from below detection limit (ND) to 8.05 mg kg^{-1} and ND to 51.21 mg kg^{-1}. Increased Pb and Cd levels were detected in the eggshells in the range of ND to 2.09 mg kg^{-1} and ND to 1.20 mg kg^{-1} (dw), respectively. No significant difference were found among the five metal contents in the eggshell in 2004, 2010, and 2014 (F = 1.88, P = 0.21 for Cu; F = 3.28, P = 0.09 for Zn; F = 0.03, P = 0.97 for Pb, and F = 0.37, P = 0.7 for Cd, F = 0.99, P = 0.41 for Ca, and F = 1.52, P = 0.27 for Mg).

The element concentrations in the internal tissues were presented in Table 4. The average Pb concentration in the kidney and liver reached 1.85 mg kg^{-1} and 1.83 mg kg^{-1}, respectively, and maximal Cd content in the liver had reached 4.42 mg kg^{-1}. The concentrations of three groups, i.e., Ca vs. Mg, Cu vs. Zn and Pb vs.

Table 3. Concentrations (mean ± SD) of six metals in the eggshell of red-crowned crane (dw, g kg^{-1} for Ca and Mg, and mg kg^{-1} for the other metals).

Year	Site	Site/size	Ca	Mg	Cu	Zn	Pb	Cd
2014	S1	S1/2	159 ± 65	0.75 ± 0.26	2.98 ± 0.58	31.31 ± 12.34	2.09 ± 0.57	0.79 ± 0.30
	S2	S2/2	239 ± 82	1.48 ± 0.51	8.05 ± 1.77	43.52 ± 18.35	0.26 ± 0.11	0.10 ± 0.02
	S3	S3/2	316 ± 134	2.39 ± 1.64	4.87 ± 0.45	21.72 ± 10.37	–	–
	S4	S4/2	177 ± 53	0.92 ± 0.33	–	–	0.72 ± 0.38	0.31 ± 0.17
	S5	S5/2	323 ± 156	1.55 ± 0.63	0.57 ± 0.38	12.15 ± 6.14	–	–
	S6	S6/2	319 ± 138	4.11 ± 1.85	0.72 ± 0.24	–	0.28 ± 0.08	0.46 ± 0.15
	S7	S72	222 ± 82	0.33 ± 0.08	1.25 ± 0.35	–	1.26 ± 0.33	–
	S8	S8/2	276 ± 71	0.84 ± 0.52	5.47 ± 1.27	23.01 ± 10.45	3.20 ± 1.05	1.20 ± 0.64
2004	S5	S5/2	311 ± 160	4.18 ± 2.27	7.37 ± 1.85	51.21 ± 16.32	1.33 ± 0.47	0.61 ± 0.26
	S8	S8/2	310 ± 128	1.51 ± 1.85	4.39 ± 0.62	15.20 ± 7.25	0.50 ± 0.19	0.12 ± 0.05
2009	S5	S5/2	127 ± 57	0.64 ± 0.34	0.56 ± 0.42	17.50 ± 5.32	0.77 ± 0.25	0.33 ± 0.11
	S7	S7/2	250 ± 62	0.79 ± 0.45	0.97 ± 0.31	6.72 ± 4.24	0.56 ± 0.16	0.45 ± 0.18
Environmental exposure level				Scarce of threshold level data			1.7 (Orlowski et al. 2010)	0.34–0.91 (Negro et al. 1993)

"–" represents the concentration was below detection level

Table 4. Tissue concentrations (mean ± SD/(min–max)) of six elements in different tissue of the breeding red-crowned crane in China (dw, g kg^{-1} for Ca and Mg, mg kg^{-1} for the other four elements; $n = 8$).

	Gut wall	Kidney	Liver
Ca	2.31 ± 1.51	1.40 ± 0.40	1.38 ± 0.42
	(0.79–4.98)	(0.81–1.70)	(1.07–2.00)
Mg	1.54 ± 1.01	1.47 ± 0.65	1.32 ± 0.41
	(0.49–2.65)	(0.79–2.31)	(0.84–1.64)
Cu	30.1 ± 10.8	42.5 ± 22.3	47.3 ± 20.5
	(15.7–39.9)	(29.5–77.5)	(28.8–72.6)
Zn	321 ± 201	370 ± 143	469 ± 198
	(129–598)	(264–577)	(209–650)
Pb	0.81 ± 0.38	1.85 ± 1.35*	1.83 ± 0.62*
	(0.09–1.39)	(0.5–2.9)	(0.38–3.21)
Cd	0.53 ± 0.34	1.23 ± 0.80	2.21 ± 1.64
	(0.12–0.96)	(0.74–2.20)	(0.37–4.42*)

Explanations: "*" indicates that the concentration exceeded the toxic level [(1.7 mg kg^{-1} for Pb, Degernes (2008); 3 mg kg^{-1} for Cd, Scheuhammer (1987)].

Table 5. Pearson correlation coefficients of the selected six elements in the eggshell of the red-crowned crane ($n = 24$).

	Zn	Pb	Cd	Ca	Mg
Cu	0.86**	0.22	−0.11	0.33	0.31
Zn		0.54	−0.53*	0.07	0.23
Pb			0.82**	−0.26	−0.27
Cd				−0.10	−0.13
Ca					0.65*

Explanations: * Correlation is significant at $P < 0.05$; ** at $P <$ 0.01 level (two-tailed).

Cd, in the eggshells exhibited significant positive correlation ($P < 0.05$), as presented in Table 5. Cd was found to be significantly negatively correlated with Zn ($P < 0.05$) and significantly positively correlated with Pb ($P < 0.01$).

Results from the principal component analysis of the six trace elements in eggshell of the red-crowned crane yielded two components that explained 95% of the variance, and three components in the internal tissue group that explained more than 90% of the variance (Table 6). For the eggshell variable, the first extracting principal component (PC1) consisted of essential elements, i.e., Cu, Zn, Ca and Mg. Six element variables were loaded in a positive direction. Pb and Cd variables were loaded as PC2, and the two loading factors had positive associations. The four other element variables were loaded in the negative direction. Three internal tissue groups, Pb and Cd, Ca and Mg, and Cu and Zn, were loaded as PC1, PC2 and PC3.

Thickness variation of the eggshells and associated with contaminants

The thickness of the eggshells varied from 605 μm to 730 μm, with an average of 663 μm (Table 7). The difference in the thickness of the eggshell were not statistically significant in 2004, 2010, and 2014 ($F = 2.93$, $P = 0.11$, Fig. 3). The average thickness of the eggshell in the buffer zone was similar to that in the core area, i.e., 682 μm versus 655 μm ($F = 1.21$, $P = 0.32$). The thin-shelled eggs presented low Ca concentrations or deficiency in Cu and Zn (Pearson $r = 0.65$, $P < 0.05$ for Ca versus thickness; Pearson $r = 0.30$, $P < 0.10$ for Mg versus thickness; Pearson $r = 0.60$, $P < 0.05$ for Cu versus thickness; and Pearson $r = 0.43$, $P < 0.05$ for Zn versus thickness). Generally, the eggs rich in Pb and Cd had thin eggshells, nevertheless, the correlations of eggshell thickness versus the toxic element concentration were pretty weak (Pearson $r = -0.32$, $P < 0.10$ for Pb versus thickness; and Pearson $r = -0.21$, $0.1 < P < 0.5$ for Cd versus thickness).

Discussion

This study focused on the trace element enrichment in the eggshells of breeding cranes and their effects on

Table 6. Component values of the principal component analysis (PCA) of different elements in the eggshells of the red-crowned crane ($n = 24$).

	Cu	Zn	Pb	Cd	Ca	Mg	Variation explained (%)
PC1	**0.95**[a]	**0.96**	0.61	0.54	**0.97**	**0.93**	72
PC2	−0.15	−0.20	**0.74**	**0.80**	−0.23	−0.35	23

Explanations: [a] Bold test indicates the variable for which each factor exhibited the greatest variability.

Table 7. Physical properties (i.e., perimeter and thickness) of the residual eggshells.

Year	Ecological zone	Site/size	Perimeter (cm)	Thickness (μm)	Thickness change (%)
2014	Buffer zone	S1/2	20.6 ± 0.5	631 ± 30	−4.96
	Buffer zone	S2/2	21.2 ± 0.4	730 ± 65	9.95
	Buffer zone	S3/2	21.7 ± 0.5	727 ± 37	−9.50
	Buffer zone	S4/2	20.6 ± 0.3	642 ± 14	−3.30
	Core area	S5/2	21.4 ± 0.4	715 ± 32	7.69
	Core area	S6/2	20.5 ± 0.5	656 ± 30	−1.19
	Core area	S7/2	20.5 ± 0.3	684 ± 20	3.02
	Core area	S8/2	21.2 ± 0.4	655 ± 22	−1.34
2004	Core area	S5/2	20.6 ± 0.5	680 ± 66	2.42
		S8/2	20.5 ± 0.6	627 ± 36	−5.56
2009	Core area	S5/2	20.3 ± 0.3	605 ± 47	−8.87
		S7/2	20.1 ± 0.6	615 ± 37	−7.36

Explanations: Thickness change = (Average thickness – Individual data)/(Average thickness) × 100%.

the eggshell thinning in China's Zhalong Wetland. The results disagreed with our initial hypothesis that increased concentrations of Pb and Cd in the prey had resulted in some essential elements, i.e., Ca, Cu and Zn deficiencies and contributed to the potential eggshell thinning in the examined species.

Generally, the maximal Pb and Cd concentrations in the sediments exceeded the probable effect level values by McDonald et al. (2000) and tolerable level for agro-economic crops by Kabata-Pendias (2001). The tissue concentrations of these metals in the corpses of red-crowned cranes were reported by Luo et al. (2015b), and the average concentrations of kidney-Pb, liver-Pb and the maximal content of liver-Cd in the red-crowned cranes exceeded the toxic level given by Degernes et al. (2008) and Scheuhammer (1987).

Comparative results of the enrichment level of metals in the eggshells of red-crowned cranes with other birds showed that Cu and Zn concentrations in the crane were similar to those in rooks in Poland (Orlowski et al. 2010), lesser kestrel in Spain (Negro et al. 1993) and yellow-breasted chat in Arizona (Mora 2003), but were significantly higher than those in the imperial eagles in Madrid (Gonzalez & Hiralda, 1998) and egret in Pakistan (Hashmi et al. 2013) (Table 8). The Pb concentrations in the red-crowned crane was similar to those of the lesser kestrel in southern Spain (Negro et al. 1993), yellow-breasted chat in Arizonan, and roseate tern of New York (Burger 1994), but were significantly higher than those in herring gull of New York (Burger 1994) and egret in Pakistan (Hashmi et al. 2013). The Cd concentrations were similar to those in rooks in Poland (Orlowski et al. 2010) and lesser kestrel in southern Spain (Negro et al. 1993), but significantly higher than those of the rest of birds. Increasing Pb and Cd concentrations in the internal tissues and eggshells of the red-crowned crane can be considered, at the very least, as indicators of potential Pb and Cd toxic risk in this species.

The results from the correlation analysis of the essential (Cu, Zn, Ca and Mg) and two nonessential (Pb and Cd) elements in the eggshells of red-crowned crane could be interoperated through chemical associations. Cd exhibited negative relations with Zn in the eggshells, which was consistent with the report by Orlowski et al. (2012b, 2014) in which Cd and Zn are primarily antagonistic in most cases. Similarly, Cu versus Zn and Pb versus Cd exhibited significantly positive correlation that could be explained by the synergistic effect of these elements.

Dauwea (2003) demonstrated that some of the Pb and Cd in the avian eggshells can also originate from external contamination, i.e., atmospheric deposition. Therefore, using avian eggshells to indicate the environmental exposure level remains a challenge. In our most recent study, we reported that the most commonly taken orders were maize, seed, and fish for the gizzard analysis, debris of zoobenthos, seed, and reed organs for the fecal analysis (Luo et al. 2015a), which were reported to contain increasing concentrations of Cd (Luo et al. 2013, 2015c). Wenzel et al. (1996) showed that the level of toxic metals in free-living birds is closely related to their concentration in the diet. Thus, we deduced that the relatively high Cd level in the eggshells

Table 8. Comparison of four elements in eggshell of the red-crowned crane with other birds (dw, mg kg^{-1}).

	Cu	Zn	Pb	Cd
Eggshell of migratory crane in China in this paper (n = 24)	3.10 ± 2.84^{ab} (ND−7.37)	18.56 ± 16.79^{a} (ND−43.52)	0.92 ± 0.93^{a} (ND−3.20)	0.33 ± 0.36^{a} (ND−1.20)
Rooks *Corvus frugilegus* in Poland (Orlowski et al. 2010, 2014)	8.14 ± 2.61^{a}	13.81 ± 2.04	3.29 ± 0.19	0.51 ± 0.01 (0.34–0.91)
Lesser kestrel *Falco naumanni* in Spain (Negro et al. 1993)	3.07 ± 2.48^{b} (0.18–7.13)	12.32 ± 4.07 (4.17–21.81)	0.89 ± 0.93 (ND–2.78)	0.71 ± 0.24^{a} (0.38–1.12)
Yellow-breasted chat in Arizona (Mora 2003)	2.5–12.5^{b}	3.3–43.2^{a}	< 0.5–1.5^{a}	–
Herring gull in New York (Burger 1994)	–	–	0.3 ± 0.05^{a}	0.05 ± 0.008^{a}
Roseate tern in New York (Burger 1994)	–	–	1.2 ± 0.3^{a}	0.10 ± 0.04^{a}
Imperial eagles in Madrid (Gonzalez & Hiraldo 1988)	0.44–9.72^{b}	6.21–9.72^{a}	0.53–1.92^{a}	0.06–0.53^{a}
Cattle egret in Pakistan (Hashmi et al. 2013)	0.01–0.31^{b}	0.06–75.5^{a}	0.05–8.55^{a}	0.05–1.9^{a}
Little egret in Pakistan (Hashmi et al. 2013)	0.06–0.31^{b}	0.53–9.27^{a}	0–4.55^{a}	0–1.6^{a}

Explanations: Values followed by the same letter in the same column means significant ($P < 0.05$). "–" means scarce of original data in these refs.

of crane was mainly due to the uptake through the daily diet.

We determined a generally consistent positive relationship for six elements in PC1, which confirmed the finding by Orlowski et al. (2014) that the sequestration of all metals into eggshells could involve their parallel co-accumulation or co-precipitation by female bird. The consistent positive correlation of the six elements in the eggshells of the red-crowned cranes in this paper could indicate that chemical interaction in the eggshell may be influenced by the intensive sequestration along the Ca binding in eggshells. The negative correlation of Cd versus Cu, Zn, and Ca in PC2 of eggshell group may be explained by the antagonistic effects of these elements.

In previous studies, deficiencies in the macronutrients Ca and Mg were found to contribute to the avian eggshell thinning (Gonzalez et al. 1988; Skalká et al. 2008; Miljeteig et al. 2012; Orlowski et al. 2014). According to Miljeteig et al. (2012), eggshell thinning degree larger than 16% would obviously induce declining of bird populations and significantly reduce their reproductive success. To the best of our knowledge, information on the critical eggshell thickness in the red-crowned crane remains scarce; this restrains the quantification of the degree of eggshell thinning in the current study. According to the previous chemical associations of the eggshells in this study, increasing Cd would reduce the Zn enrichment. Nevertheless, we did not find significant correlations between Cd and Ca contents. The correlations of Pb vs. eggshell thickness and Cd vs. eggshell thickness were also weak. Therefore, certifying whether the risk of exposure to Pb and Cd are the principal contributors to the observed bird population declines is challengeable (Luo et al. 2015c). However, the detectable Pb and Cd in the body and eggshells of this species should receive significant attention by the management department in Zhalong Wetland, and heavy metal inputs into the wetland should be reduced to ensure that this critical crane habitat will remain in a healthy state for the long-term sustainability of the crane population.

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