Evaluation of the lead exposure situation in wild birds and identification of novel avian renal biomarkers

国内に生息する鳥類における鉛汚染状況の解明と、
鳥類種横断的に利用可能な新規腎障害マーカーの探索

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CONTENTS

CHAPTER 2

Lead exposure in raptors and source identification using lead isotope ratios

CHAPTER 3

Current situation of lead exposure in birds in Japan (2015-2017); lead exposure is still occurring

CHAPTER 5

Discovery of novel renal biomarkers in a chicken model using a glycomic approach

CHAPTER 6

Discovery of novel renal biomarkers in a chicken model using transcriptome analysis

ABBREVIATIONS

AAALAC: Association for the Assessment and Accreditation of Laboratory Animal Care International

AAS: atomic absorption spectrometry

ad.: adult

AIN: acute interstitial nephritis

AKI: acute kidney injury

ANLN: anillin actin binding protein

As: arsenic

AST: aspartate aminotransferase

ATN: acute interstitial nephritis

BOA: benzyloxyamine

BW: body weight

C: carbon

Ca: calcium

Cd: cadmium

cDNA: complementary DNA

ch.: chick

CK: creatine phosphokinase

Co: cobalt

Cont.: control

cps: count per second

Cr: chromium

Ct: cycle threshold

Cu: copper

DAVID: Database for Annotation, Visualization and Integrated Discovery

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DW: distilled water

EDTA: ethylenediaminetetraacetic acid

emb.: embryo

f.: female

Fe: iron

FSGS: focal segmental glomerulosclerosis

G: gauge

GFR: glomerular filtration rate

GO: Gene Ontology

GRP: gastrin releasing peptide

HCL: hydrochloric acid

HCT: hematocrit

Hg: mercury

HNO_3: nitric acid

HPLC: high-performance liquid chromatography

Ht: hematocrit

ICP-MS: inductively coupled plasma–mass spectrometer

ICP-QQQ-MS: triple quadrupole inductively coupled plasma–mass spectrometer

IRBJ: Institute for Raptor Biomedicine Japan

IUCN: International Union for Conservation of Nature

JICA: Japan International Cooperation Agency

JST: Japan Science and Technology Agancy

juv.: juvenile

KEGG: Kyoto Encyclopedia of Genes and Genomes

KIM-1: kidney injury molecule-1

LA-ICP-MS: laser ablation inductively coupled plasma–mass spectrometer

LDH: lactate dehydrogenase

m.: male

MALDI-TOF/MS: matrix-assisted laser desorption ionization, time-of-flight mass spectrometry

MCM10: minichromosome maintenance complex component 10

MCV: mean corpuscular volume

MEGA: Molecular Evolutionary Genetics Analysis

Mg: Magnesium

MeHg: methylmercury

Mn: manganese

MT: metallothionein

N.A.: not available

NCBI: National Center for Biotechnology Information

N.D.: not detectable

NGAL: neutrophil gelatinase-associated lipocalin

Ni: nickel

NIST: National Institute of Standards and Technology

NSAID: non-steroidal anti-inflammatory drug

OAT: organic anion transporter

P: inorganic phosphate

PAH: *p*-aminohippuric acid

PAS: Periodic acid-Schiff stain

Pb: lead

PCA: principal component analysis

PCR: polymerase chain reaction

PKG1: phosphoglycerate kinase 1

PNGase F: Peptide-N-Glycosidase F

PTFE: polytetrafluoroethylene

RBC: red blood cell

RCC: renal cell carcinoma

RNA: ribonucleic acid

RSD: relative standard deviation

SD: standard deviation

sub.: sub-adult

TPP: total plasma protein

UA: uric acid

UNEP: United Nations Environment Programme

unk.: unknown

UV: ultraviolet

VNN1: vanin-1

WBC: white blood cell

wt: weight

YWHAZ: yrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

Zn: zinc

PREFACE

Importance of wildlife conservation

"One World - One Health" is a new concept that human, animal, and environmental health are all inextricably linked. There are many aspects to this important concept, and wildlife conservation is strongly needed as one of the approaches to "One Health."

According to the Red List published by IUCN (International Union for Conservation of Nature) in 2017, the number of highly endangered bird species is 1460. This number is larger than that of mammals (1196 species) and reptiles (1185 species), and many of these birds are threatened with extinction (IUCN, 2017). In Japan, 33 species of mammals and 97 species of birds were designated as endangered species in the Red List published by the Ministry of the Environment in 2017. There are many endangered umbrella species and keystone species that are highly linked with maintaining the ecosystem. Conservation is highly important from two sides: protection for the endangered species itself and preservation of biodiversity.

Reductions in wildlife populations have numerous causes, such as environmental pollution, climate change, infectious diseases, habitat loss caused by development, impact of invasive alien species, and poaching. It is a serious problem that many cases have been caused by human activities. Therefore, wildlife conservation is our responsibility.

Serious damage to birds caused by chemical pollution

As the Red Lists show, a decrease in the number of birds has been a serious

problem in recent years. Infectious diseases such as avian influenza can be cited as one cause of the decreasing number of birds, but chemical contamination is also a remarkable problem. Damage caused by pollution with metals, oil, and agricultural chemicals has been reported all over the world, and chemical contamination sometimes leads to mass deaths. For example, the California condor (*Gymnogyps californianus*) population in the United States has drastically decreased due to lead (Pb) poisoning (Finkelstein et al., 2012), and three species of vultures on the Indian subcontinent have declined by more than 95% due to secondary ingestion of diclofenac (Oaks et al., 2004; Green et al., 2004; Swan et al., 2006). Not only is this important from the viewpoint of species conservation, but the reduction of bird of prey species, which are located at the top of the food chain, will have an impact on the entire ecosystem, and therefore conservation of these species is an urgent issue.

Seabirds are top predators in ocean ecosystems and there is a concern that they are at high risk of bioconcentrated chemical contamination. In the ocean, contamination by metals such as mercury (Hg), cadmium (Cd), and arsenic (As) is a concern, and ocean pollution is increasing, particularly with respect to Hg, as reported by UNEP (United Nations Environment Programme) (UNEP 2013).

In raptors and waterbirds, Pb exposure is also a serious form of environmental pollution (Ochiai et al., 1993; Saito, 2009). Pb rifle bullets, Pb shot pellets, and fishing tackle are the main sources of Pb exposure in birds. Raptors take up Pb ammunition and are thus exposed to Pb when they eat prey such as sika deer, wild boar, and birds that have been shot with Pb ammunition. Pb bullets are broken into pieces when they enter the bodies of hunted animals, so fragments of Pb remain in many parts of the body, and birds of prey begin to eat from the wounded part, where the fragments are most concentrated.

Therefore, raptors have a high risk of ingesting Pb bullets. Waterbirds are exposed to Pb because they swallow pebbles as stomach stones, and may mistakenly ingest Pb shot or fishing tackle that has fallen into lakes and ponds. In addition, if birds of prey consume waterbirds that were exposed to Pb, the birds of prey may suffer secondary Pb pollution damage. It has been reported that one grain of Pb shot contains an amount of Pb that is lethal to birds when ingested (Pain and Rattner, 1988)

A large number of kidney injuries in birds due to their unique kidney structure

Many chemical substances, such as anti-inflammatory drugs, anti-cancer drugs and heavy metals cause kidney damage in birds. One of the reasons is a special vascular system called the renal portal vein (Harr, 2002), that dose not exist in mammals, and there is high amount of chemical exposure to the kidney via the blood.

On the Indian subcontinent, over 95% of the population of three vulture species (oriental white-backed vulture [*Gyps. bengalensis*], Indian vulture [*G. indicus*] and the slender-billed vulture [*G. tenuirostris*]) have died from renal injury caused by secondary ingestion of diclofenac, an anti-inflammatory drug administered to livestock (Oaks et al., 2004; Green et al., 2004; Swan et al., 2006). Pb poisoning is reported to cause severe kidney damage in raptors and waterbirds (Johnson, 1998). Many medicines used to treat birds (chelating agents used for Pb poisoning, antifungal agents used for aspergillosis, anti-cancer drugs, anti-inflammatory drugs, and more) also have side effects of kidney damage (Filippich et al., 2001; Joseph, 2000). As mentioned above, renal disorders due to these nephrotoxic chemical substances have been reported in many wild birds.

However, it is currently impossible to detect early kidney injury in birds using

blood biochemical examinations. Currently, the uric acid value is used in diagnosis and treatment, but the uric acid level is not changed unless renal function is decreased by more than 70%, and in most cases renal function cannot be expected to recover at that point (Lierz, 2003). For example, in the Okinawa rail (*Gallirallus okinawae*), if an increase in uric acid level is observed, the individual is likely to die within a week even if treatment is given. In other avian species, a rise in uric acid levels in blood tests also often means death. In birds of prey, however, the uric acid value fluctuates greatly before and after meals, so the uric acid level is not a good marker for kidney injury (Lierz, 2003). Therefore, identification of novel renal biomarkers in birds is required.

Our research results have potential applications in translational research and the clinical use of a wide variety of avian species, ranging from rare wild birds to companion birds, and even reptiles with similarities in internal structure. There are many endangered species of birds, and the possibility of developing new methods in rescue and conservation of birds and reptiles, which are difficult to examine, is predicted to have a great impact on a global scale.

Structure of this paper

This paper consists of six chapters.

Chapter 1: Contamination status of metals and arsenic in seabirds from the Bering Sea and Teuri Island

Because of the abundance of food resources in the Bering Sea, which lies along

the margin of the Pacific Ocean, many seabirds and other marine animals gather there (Overland and Stabeno, 2004). The massive gathering of animals is called “Aleutian Magic.” On the other hand, Teuri Island, located in Hokkaido, is the world’s largest breeding ground of rhinoceros auklets (*Cerorhinca monocerata*): about 400,000 pairs of rhinoceros auklets visit the island for breeding every year. Although many avian species inhabit these areas, information about metal accumulation in these seabirds is scarce.

In this study, I focused on the above two sites, which are important habitats for seabirds. In order to elucidate the status and characteristics of metal contamination and accumulation in seabirds living in these places, contamination status was investigated in seabirds from both the Bering Sea and Teuri Island. Characteristics of metal accumulation in different seabird species were clarified.

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- Ishii, Chihiro, et al. "Contamination status and accumulation characteristics of heavy metals and arsenic in five seabird species from the central Bering Sea." *Journal of Veterinary Medical Science* 79.4 (2017): 807-814.
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Chapter 2: Lead exposure in raptors and source identification using lead isotope ratios

Chapter 3: Current situation of lead exposure in birds in Japan (2015-2017); lead exposure is still occurring

Chapter 4: Lead distribution in bones of lead-poisoned eagles and swans; bone samples as useful indicators

In chapter 1, it is shown that seabirds accumulate high concentrations of metals. In Japan, metal contamination, especially Pb exposure, is a serious problem in birds of prey and waterbirds (Ochiai et al., 1993; Saito, 2009). Many birds have died due to Pb poisoning, and therefore I focus on this matter in chapters 2, 3, and 4.

In Hokkaido, the use of Pb ammunition in hunting is banned because a large number of endangered eagles (Steller's sea eagle [*Haliaeetus pelagicus*] and white-tailed sea eagle [*Haliaeetus albicilla*]) died due to the accumulation of high levels of Pb. However, Pb poisoning has occurred every year in these rare eagles. In the area south of Honshu, Pb-use restrictions are limited to some areas such as the wetlands protected by the Ramsar Convention. Surveys of Pb contamination in birds are rarely conducted, and the actual situation has not been clarified. Therefore, in this research, I attempted to elucidate the actual Pb exposure in birds of prey and waterbirds and to identify the sources of Pb by using Pb stable isotope ratios.

The Pb absorbed in the body is carried by the blood to organs throughout the body. It causes hematotoxicity, neurotoxicity, and toxicity in various organs including the liver and kidney, eventually leading to death.

Because the final accumulation site of Pb is bone (Fisher et al., 2006) and the structure of bone varies depending on its location, it is thought that information useful for Pb contamination assessment can be obtained by examining various bones. Bones are the body parts most likely to remain in nature and are also stored in museums and the like, so they are highly useful. Based on these backgrounds, in order to clarify the Pb contamination damage in birds across Japan, bone samples from throughout the bodies of

eagles and swans were collected and Pb distribution in the bones was analyzed.

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Chapter 5: Discovery of novel renal biomarkers in a chicken model using a glycomic approach

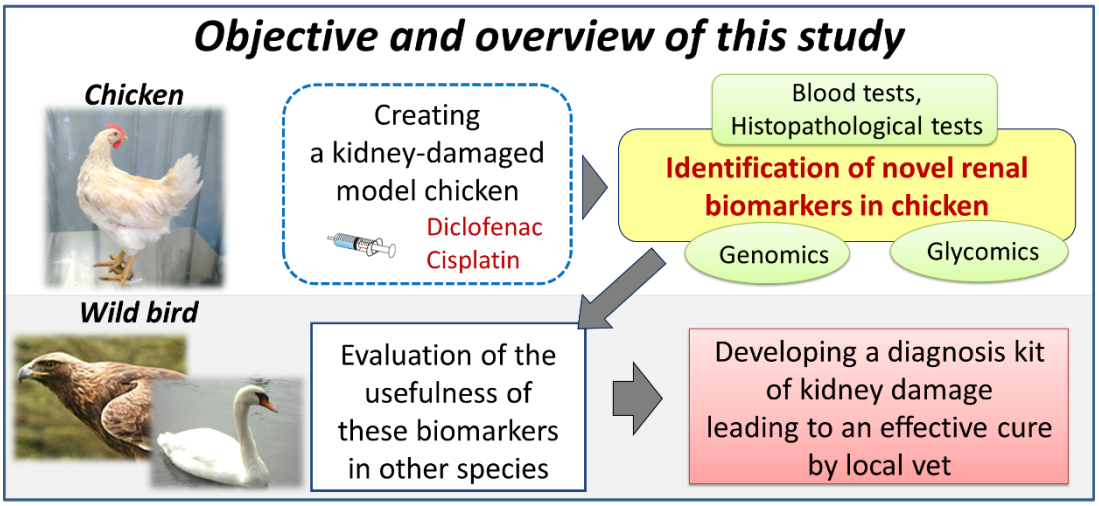
Chapter 6: Discovery of novel renal biomarkers in a chicken model using transcriptome analysis

In this research, the objective was to identify novel biomarkers and develop a kidney injury diagnosis kit that will be effective in actual clinical settings, to treat renal impairment, monitor administration of drugs that can cause renal impairment, and evaluate environment-based contamination by chemical substances that cause kidney damage. The ultimate goal is a contribution to avian conservation through translational research, from basic research to clinical application. First, I created renal disorder models in chickens and searched for candidate markers. Then, blood samples from wild birds, both healthy birds and those with possible kidney injuries, will be used to confirm the usefulness of the candidate markers.

Cisplatin, an anti-cancer drug that causes renal tubular necrosis, and diclofenac,

a nonsteroidal anti-inflammatory drug that causes interstitial nephritis, were administered to chickens to create different types of kidney injury models.

Kidney-specific disorders were confirmed by blood biochemical test and pathological examination, and in order to select nephropathy marker candidates, analysis of plasma *N*-glycans (comprehensive analysis using the glycoblotting method) and transcriptome analysis of kidney samples (selection of candidate genes by exhaustive analysis using microarrays and quantitative real-time PCR analysis of each gene) were carried out. Glycomic analysis has attracted attention in the field of human medicine in recent years, and uses sugar chains as an early diagnostic marker of diseases such as cancer. Transcriptome analysis has been used for disease-marker searching.



If a novel kidney injury marker in birds could be identified by this research, and a simple kidney injury diagnosis kit designed, it would be useful for (1) early detection of renal disorders in birds, (2) monitoring treatment with medicines that cause nephrotoxicity as a side effect, such as therapeutic drugs for Pb poisoning, and (3) early

detection of chemical contamination that causes renal disorders.

For an example of (1), in many species of birds, when the uric acid value, a conventional renal impairment marker often used as an index, rises, 70% or more of renal function is already lost, and treatment will not be successful. The discovery and use of an early marker would make it possible to find kidney disease when there is a low degree of disability. If it becomes possible to diagnose early-stage renal disorders in birds, it will be possible to treat a wide variety of renal disorders in a wide variety of species, ranging from rare birds to companion birds. This can contribute to avian conservation.

Regarding (2), chelating agents used for treatment of Pb poisoning in birds of prey and waterbirds, and antifungal drugs used for aspergillosis, which is more common in seabirds, are representative of the medicines that cause renal damage as a side effect. It is reported that nonsteroidal anti-inflammatory drugs and anti-cancer drugs have renal side effects in mammals. Avian species have greater blood flow to the kidney than mammals do, so renal side effects of these therapeutic agents are more likely than in mammals. One problem actually occurring in clinical practice, for example, is that chelating agents used for detoxification of Pb-poisoned individuals may cause side effects, so even in the case of serious symptoms caused by Pb, a structured treatment interruption is needed. Thus, therapeutic drugs with renal impairment effects must be used with caution, while considering the balance between the original disease and side effects. By monitoring kidney status using early renal injury markers, side effects on the kidneys can be detected at an early stage, therapeutic drugs can be used more appropriately, and the treatment effects can be expected to improve accordingly.

As for (3), although there are many chemical substances that cause kidney damage in wild birds, including Pb, Cd, Hg, and drugs that are diffused in the environment,

actual damage cannot clearly be identified without analyzing the carcasses. Regarding the specific toxicological effects on birds, it is almost unknown which concentrations of various chemical substances adversely affect the living body because of the large differences in susceptibility among species. Therefore, if it becomes possible to evaluate exposure using a sample that can be collected almost noninvasively, such as blood, there is a possibility that exposure to environmental pollutants can be detected, and there is an opportunity to evaluate and grade the damage. If an appropriate contamination assessment is made before the pollution damage spreads throughout the body, it is possible to take countermeasures directly against the chemical substance and initiate appropriate treatment for the damaged individual. For example, in India, more than 95% of three species of vultures died due to the secondary intake of diclofenac, an anti-inflammatory agent administered to domestic animals on the Indian subcontinent; this is an example of how chemical contamination in the environment can cause enormous damage. Investigation of the causes of damage is very important in reducing the cases of damage or death and for conservation of wild birds.

This content was announced in the following journal.

- Ishii, Chihiro, et al. "A glycomics approach to discover novel renal biomarkers in birds by administration of cisplatin and diclofenac to chickens." *Poultry Science*. In press. 2017

References

IUCN:http://cmsdocs.s3.amazonaws.com/summarystats/2017-2_Summary_Stats_Page_Documents/2017_2_RL_Stats_Table_1.pdf

Church, M.E., Gwiazda, R., Risebrough, R.W., Sorenson, K., Chamberlain, C.P., Farry, S., Heinrich, W., Rideout, B.A., Smith, D.R., 2006. Ammunition is the principal source of lead accumulated by California condors re-introduced to the wild. Environ. Sci. Technol. 40, 6143–6150.

Filippich, L.J., Bucher, A.M., Charles, B.G., Sutton, R.H., 2001. Intravenous cisplatin administration in sulphur-crested cockatoos (*Cacatua galerita*): Clinical and pathologic observations. J. Avian Med. Surg. 15, 23–30.

Finkelstein, M.E., Doak, D.F., George, D., Burnett, J., Brandt, J., Church, M., Grantham, J., Smith, D.R., 2012. Lead poisoning and the deceptive recovery of the critically endangered California condor. Proc. Natl. Acad. Sci. 109, 11449-11454. doi:10.1073/pnas.1203141109

Fisher, I.J., Pain, D.J., Thomas, V.G., 2006. A review of lead poisoning from ammunition sources in terrestrial birds. Biol. Conserv. 131, 421–432. doi:http://dx.doi.org/10.1016/j.biocon.2006.02.018

Green, R.E., Newton, I.A.N., Shultz, S., Cunningham, A.A., Gilbert, M., Pain, D.J., Prakash, V., 2004. Diclofenac poisoning as a cause of vulture population declines across the Indian subcontinent. J. Appl. Ecol. 41, 793–800.

Harr, K.E., 2002. Clinical chemistry of companion avian species: a review. Vet. Clin. Pathol. 31, 140–151.

Johnson, F.M., 1998. The genetic effects of environmental lead. Mutat. Res. Mutat. Res. 410, 123–140.

Joseph, V., 2000. Aspergillosis in raptors, in: Seminars in Avian and Exotic Pet Medicine. Elsevier, pp. 66–74.

Lierz, M., 2003. Avian renal disease: pathogenesis, diagnosis, and therapy. Vet. Clin. North Am. Exot. Anim. Pract. 6, 29–55.

Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad, H.L., Ahmed, S., Iqbal Chaudhry, M.J., Arshad, M., Mahmood, S., Ali, A., Ahmed Khan, A., 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. Nature 427, 630–633.

Ochiai, K., Hoshiko, K., Jin, K., Tsuzuki, T., Itakura, C., 1993. A survey of lead poisoning in wild waterfowl in Japan. J. Wildl. Dis. 29, 349–352.

Overland, J.E., Stabeno, P.J., 2004. Is the climate of the Bering Sea warming and affecting the ecosystem? Eos, Trans. Am. Geophys. Union 85, 309–312. doi:10.1029/2004EO330001

Pain, D.J., Rattner, B.A., 1988. Mortality and hematology associated with the ingestion of one number four lead shot in black ducks, *Anas rubripes*. Bull. Environ. Contam. Toxicol. 40, 159–164.

Saito, K., 2009. Lead poisoning of Steller's Sea-Eagle (*Haliaeetus pelagicus*) and Whitetailed Eagle (*Haliaeetus albicilla*) caused by the ingestion of lead bullets and slugs. Hokkaido Japan. RT Watson, M. Fuller, M. Pokras, WG Hunt (Eds.). Ingestion Lead from Spent Ammunit. Implic. Wildl. Humans. Peregrine Fund, Boise, Idaho, USA.

Scheuhammer, A.M., Templeton, D.M., 1998. Use of stable isotope ratios to distinguish sources of lead exposure in wild birds. Ecotoxicology 7, 37–42.

Swan, G.E., Cuthbert, R., Quevedo, M., Green, R.E., Pain, D.J., Bartels, P.,

Cunningham, A.A., Duncan, N., Meharg, A.A., Oaks, J.L., 2006. Toxicity of diclofenac to Gyps vultures. Biol. Lett. 2, 279–282.

UNEP, 2013. Global Mercury Assessment 2013: Sources, Emissions, Releases and Environmental Transport. UNEP Chemicals Branch, Geneva, Switzerland

CHAPTER 1

Contamination status of metals and arsenic in seabirds from the Bering Sea and Teuri Island

Abstract

As marine top predators, seabirds accumulate high levels of metals and metalloids in their tissues through the consumption of contaminated prey. The Bering Sea is home to many seabirds, pinnipeds, and whales, and Teuri Island in Hokkaido, Japan, is a key breeding site for seabirds. Therefore, the aim of this study was to determine the levels of metal contamination in seabirds from these sites. The concentrations of eight heavy metals (mercury [Hg], cadmium [Cd], chromium [Cr], cobalt [Co], nickel [Ni], copper [Cu], zinc [Zn], and lead [Pb]) and one metalloid (arsenic [As]) were measured in the livers and kidneys of seabirds collected from these areas, and the nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) stable isotope ratios in the breast muscles of the birds were also determined as indicators of the trophic level and habitat of each species. In the Bering Sea, northern fulmars (*Fulmarus glacialis*) had high hepatic Hg concentrations, while both northern fulmars and tufted puffins (*Fratercula cirrhata*) had high Cd levels. There was also a positive correlation between the Hg concentrations and $\delta^{15}N$ values across individual birds, suggesting that Hg uptake is linked to the trophic status of the consumed prey, and Hg concentrations were higher than was detected in the same species of seabirds collected in 1990. On Teuri Island, spectacled guillemots (*Cepphus carbo*) had high As concentrations, which originated from their feeding habitat, while rhinoceros auklets (*Cerorhinca monocerata*) had high concentrations of Hg. The findings for Teuri Island will serve as useful baseline data for future comparisons.

Keywords

Bering Sea, Teuri Island, seabird, mercury, cadmium

Highlights

- There is a positive correlation between Hg concentrations and $\delta^{15}N$ values across individual birds, suggesting that Hg uptake is linked to the trophic status of the consumed prey.
- Seabirds in the Bering Sea have higher Hg concentrations than were detected in the same species in 1990.
- The findings for Teuri Island will serve as useful baseline data for future comparisons.

1. Introduction

There is increasing concern that seabirds, which are marine top predators, are accumulating high concentrations of heavy metals such as lead (Pb), mercury (Hg), and cadmium (Cd) in their tissues, which can result in reduced reproductive success and survival (Burger, 2008; Elliott et al., 1992; Lucia et al., 2010; Watanuki et al., 2015).

The Bering sea is a northern extension of the Pacific Ocean that provides important habitat for various animals, including 80% of seabirds in the USA (Overland and Stabeno, 2004), due to the abundance of food. However, although information on metal accumulation is available for many avian species, little is known about those living offshore (Honda et al., 1990).In addition, many birds migrate to the large number of islands in Japan, with Teuri Island in Hokkaido being the largest breeding ground in the world for rhinoceros auklet (*Cerorhinca monocerata*) and an important breeding site for the common murre (*Uria aalge*). However, organized research on metal and metalloid pollution has been suspended in this region.

Hg pollution is of particular concern in the marine environment. The ocean receives approximately 90% of its Hg through wet and dry atmospheric deposition (Mason et al., 1994), and anthropogenic activities have a significant effect on the global Hg cycle (Fitzgerald et al., 2007), with fossil fuel-fired power plants, artisanal small-scale gold mining, and non-ferrous metals manufacturing making major contributions (Pirrone et al., 2010)–indeed, UNEP (2013) reported that marine animals accumulated 10–12 times more Hg in 2013 than in pre-industrial times. Aquatic ecosystems are major repositories of natural and pollution-derived Hg, and so wildlife species that have a primarily marine-based diet are at greater risk of metal exposure in their feeding habitats. Furthermore, these ecosystems also host active populations of Hg methylating bacteria, making them

particularly susceptible to monomethylmercury contamination (Fitzgerald et al., 2007). Methylmercury (MeHg) has greater toxicity and bioaccumulation than Hg itself, and can pass through the blood–brain barrier to cause brain failure, central nervous system dysfunction, and spinal cord degeneration (Wolfe et al., 1998).

The aim of this study was to determine the levels of metal contamination in seabirds inhabiting the central Bering Sea and Teuri Island. To do this, the accumulation patterns of eight heavy metals (Hg, Cd, chromium [Cr], cobalt [Co], nickel [Ni], copper [Cu], zinc [Zn], and Pb) and one metalloid (arsenic [As]) were examined in the livers and kidneys of seabirds from this area. Since some heavy metals are known to be biomagnified through the food chain (Atwell et al., 1998) and to vary across habitats (Larison et al., 2000), nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) stable isotope ratios in the muscles of these seabirds were also measured as indicators of their trophic levels and habitats.

2. Materials and Methods

2. 1. Sampling

From the central Bering Sea (44°00′N – 57°30′N, 178°00′E – 177°00′W, Fig. 1.), specimens were sampled from seabirds entangled in gill nets during the Japan–US joint research on salmon in June or July from 2008 to 2010. The liver and kidneys were collected from short-tailed shearwater (*Puffinus tenuirostris*) (n = 24), tufted puffin (*Fratercula cirrhata*) (n = 5), northern fulmar (*Fulmarus glacialis)* (n = 4), thick-billed murre (*Uria lomvia*) (n = 4), and horned puffin (*Fratercula corniculata*) (n = 3). From Teuri Island (44°25′N, 141°52′E, Fig. 2.), located in the Japan Sea of Hokkaido, liver and kidney samples from slaty-backed gull (*Larus schistisagus*) (n = 15), rhinoceros auklet (*Cerorhinca monocerata*) (n = 7), spectacled guillemot (*Cepphus carbo*) (n = 6) and thick-billed murre (*Uria lomvia*) (n = 2) were collected from May to July in 2012 and 2013. Specimens of liver and kidney were used for the metals and metalloid analysis. Breast muscles of short-tailed shearwater (n = 23), slaty-backed gull (n = 15), northern fulmar (n = 4), thick-billed murre (n = 3), tufted puffin (n = 3) and horned puffin (n = 2) were also collected for the $\delta^{13}C$ and $\delta^{15}N$ analyses. All samples were kept at –20°C in a freezer until analysis.

2.2. Extraction and analysis of metals and a metalloid

Analyses were performed according to the method of Yabe *et al*. (Yabe et al., 2013). For metal analysis, 0.3 g of liver and kidney samples were dried for 15 hr at 50°C and then digested with 6 mL of 60% nitric acid (Kanto Chemical Corporation, Tokyo, Japan) and 1 mL of 30% hydrogen peroxide (Kanto Chemical Corporation) in a microwave digestion system (Speedwave Two; Berghof, Eningen, Germany). Digestion

was performed under the following conditions: 180°C for 15 min, 200°C for 20 min and 100°C for 20 min. After the samples were cooled, they were transferred to plastic tubes into which was added 0.1 mL of lanthanum chloride (Wako Pure Chemical Industries, Osaka, Japan). The volume was then brought to 10 mL with 2% nitric acid. The concentrations of heavy metals and a metalloid (Cd, Cr, Co, Ni, Cu, Zn, Pb and As) were measured using an atomic absorption spectrophotometer (AAS) (Z-2010; Hitachi High-Technologies Corporation, Tokyo, Japan) with acetylene flame or argon non-flame method. The instrument was calibrated using standard solutions of the respective heavy metals to establish standard curves before analysis. Concentrations of Cu and Zn were determined through the flame method with acetylene gas, whereas concentrations of Cd, Cr, Ni, Pb and As were determined using a graphite furnace with argon gas. All chemicals and standard stock solutions were of analytical reagent grade (Wako Pure Chemicals Industries). Water was distilled and deionized (Milli-Q; Merck Millipore, Billerica, MA, USA). Analytical quality control was performed using DOLT-4 (dogfish liver) and DORM-3 (fish protein) certified reference materials (both from the National Research Council of Canada). Recovery rates (%) of all elements were acceptable: Cd (91 – 108), Cr (91 – 108), Co (96 – 111), Ni (98 – 111), Cu (88 – 90), Zn (78 – 83) and Pb (89 – 98). Arsenic had lower recovery rates (50% – 67%). Detection limits (μg/kg) for Cd, Cr, Co, Ni, Cu, Zn, Pb and As were 0.2, 0.5, 0.5, 0.5, 1.0, 0.1, 1.0 and 2.0, respectively.

2.3. Analysis of total mercury (Hg)

The concentrations of total Hg in the liver and kidneys were measured by thermal decomposition, gold amalgamation and atomic absorption spectrophotometry using a mercury analyzer (MA-3000; Nippon Instruments Corporation, Tokyo, Japan), after

preparation of the calibration standard. The recovery rates of Hg for the certified reference material, DOLT-4, ranged from 92% to 103%. The recovery rate of Hg was 94.3% ± 4.2%. The concentration of Hg was converted from mg/kg wet weight to mg/kg dry weight using the calculated water content of specimens before and after drying.

2.4. Stable isotope ratio analysis ($\delta^{13}C$ and $\delta^{15}N$)

Stable isotope ratio analysis was performed according to the method of Nakayama *et al*. (Nakayama et al., 2013). Muscle tissue has longer turnover time than liver and is the most common samples used for stable isotope analysis (Post et al., 2007). Muscles were washed with distilled water to remove blood and then dried at 45°C for 48 hr. Samples were then ground into a homogeneous powder and treated with a 2:1 chloroform–methanol solution (Kanto Chemical Corporation) to remove lipids, and the residue was dried. Each sample was weighed (0.5 – 1.0 mg) into a tin capsule (Säntis Analytical AG, Teufen, Switzerland), and stable isotope ratios were determined by the flow injection method using a Finnigan MAT-252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany) connected to a Fisons NA1500 elemental analyzer (Fisons Instruments SpA, Strada Rivoltana, Italy). Stable isotope ratios were expressed in δ notation (as deviation from standards in parts per thousand (‰)) according to the following formula: $\delta X = [(R_{sample}/R_{standard} - 1)] \times 1{,}000$, where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$ (Minagawa and Wada, 1984). Data are presented as values based on the international standard of v-PDB (Vienna Peedee Belemnite; fossilized shells from the PeeDee Formation in South Carolina) and as atmospheric N_2 for C and N, respectively (Lucia et al., 2010). Replicate errors were within 0.2‰ for both $\delta^{13}C$ and $\delta^{15}N$ analyses.

2.5. Statistical analysis

Differences in the metal and arsenic concentrations and isotope ratio values among species were examined using Tukey's honest significant difference (HSD) test, with separate analyses for birds from the Bering Sea and Teuri Island. Thick-billed murre was excluded from the Teuri Island analysis due to the small sample size (n = 2). In addition, differences in the levels of metal and arsenic accumulation between the sexes and different age groups of short-tailed shearwater (*Puffinus tenuirostris*) were examined using the Kruskal-Wallis test, because the sample information and sample size were sufficient for more detailed analysis in this species. The Spearman's rank correlation coefficient (r) and principal component analysis (PCA) were then used to analyze the relationships between pollutant concentration, body weight, and stable isotope ratio. All analyses were performed in JMP Pro 10.0.2 (SAS Institute, Cary, NC, USA) with a significance level of $p < 0.05$.

3. Results

3.1. Metal and arsenic accumulation

The sample information, stable isotope ratios, and metal and arsenic concentrations in seabirds from the Bering Sea are shown in Tables 1 and 2, while the levels of metal and arsenic accumulation in seabirds from Teuri Island are shown in Table 3. The results indicated that the seabirds examined in this study had high concentrations of Hg and Cd compared with herbivorous or insectivorous terrestrial bird species (Alleva et al., 2006).

The accumulation patterns of metals differed among species at both sites. In the Bering Sea, northern fulmar had significantly higher Hg concentrations in the liver than the other species (Table 2), while northern fulmar and tufted puffin accumulated higher concentrations of Cd. There were no differences in the concentrations of Co, Ni, and As in the liver, and Cr, Cu, and Pb in the liver and kidney among species, however.

On Teuri Island, spectacled guillemot accumulated higher concentrations of As in both the liver and kidney than the other species (Table 3), with one specimen having a nephrotic Cd level. In addition, rhinoceros aucklet accumulated higher concentrations of Hg than other species in the family Alcidae (thick-billed murre and spectacled guillemot), as well as short-tailed shearwater, tufted puffin, and horned puffin (Table 3).

3.2. Relationship between metal and arsenic accumulation, trophic level, and feeding habitat

There were strong positive correlations between the Hg concentrations in the liver and $\delta^{15}N$ values in the muscle (Table 5), and positive correlations between the concentrations of As and Cu, and Zn and Cd ($r > 0.5$). In addition, body weight was

positively correlated with $\delta^{13}C$ values but negatively correlated with the concentrations of Cr and Cu, and $\delta^{13}C$ values were also negatively correlated with Cr concentrations. PCA analysis showed that these characteristics varied between species (Fig. 3).

3.3. Sex and age differences in metal and arsenic accumulation in *short-tailed shearwater*

Male short-tailed shearwaters accumulated significantly higher Hg concentrations in their livers than females, whereas females accumulated higher levels of Cd and Pb. Furthermore, adult short-tailed shearwaters had higher levels of Cr, whereas juveniles had higher levels of Co, Cu, $\delta^{13}C$, and $\delta^{15}N$ (Table 6).

4. Discussion

4. 1. Relationship between metal concentrations and the stable isotope ratios

In this study, the $\delta^{15}N$ level was used to indicate the trophic level of each species, which showed that northern fulmar is at a higher trophic level than the other species examined. Northern fulmars eat various fish and squid species (Table 4), whereas horned puffins mainly eat small fish (Fitzgerald et al., 2007). In addition, there were high correlations among $\delta^{15}N$, $\delta^{13}C$ and Hg levels, indicating that both the trophic level and Hg accumulation may reflect these feeding habitats.

There were significant differences in Hg concentration between northern fulmar and the other species examined, suggesting that Hg uptake is linked to the trophic status of the prey consumed. It is generally accepted that Hg is a ubiquitous environmental contaminant that is typically transferred through aquatic and terrestrial food webs (Blevin et al., 2013; Bryan Jr. et al., 2012). The high $\delta^{15}N$ value for northern fulmar indicated its position as a top predator in the food chain, and thus its higher risk of toxicity from Hg and other persistent pollutants compared with other bird species. Furthermore, individuals of this species have a long life span, meaning that they can accumulate Hg over long periods (Ricca et al., 2008).

There was a strong positive correlation between the concentrations of Cd and Zn among individuals, which is consistent with previous reports (Honda et al., 1990; Scheuhammer, 1987). These heavy metals are expected to interact with detoxification systems such as metallothionein (MT), a low-molecular-weight cysteine-rich protein that is involved in the homeostasis of essential metals such as Zn. The synthesis of MT can be induced by heavy metals such as Cd, Zn, Cu, and inorganic Hg, and so concentrations of Cd and MT are positively correlated in many seabirds (Elliott et al., 1992; Stewart et al.,

1996). However, high exposure to Cd will also increase Zn uptake, as this is essential for the synthesis of MT. Supporting this, it has previously been reported that Cd exposure increases the concentrations of Zn and MT mRNA in the kidneys and livers of rats (Zhang et al., 2012). In birds, MT can be synthesized or degraded rapidly in response to changing concentrations of metals (seasonally or through molting), which likely explains the Cd–Zn relationship (Zhang et al., 2012).

4.2. Sex and age differences in the metal and arsenic concentrations and stable isotope ratios in short-tailed shearwater

Sex differences in metal accumulation might be expected if males and females eat different foods, different-sized foods, or different proportions of various foods (Burger, 1995). Furthermore, it has been shown that females can reduce the amount of Hg that is transferred to their eggs (Robinson et al., 2011).

Female short-tailed shearwaters had significantly higher concentrations of Cd and Pb than males. Similarly, it has previously been reported that female black skimmers (*Rynchops niger*) accumulate higher levels of Cd and Pb in their breast feathers than males, possibly due to differences in the species composition of the diet (Burger, 1995). The present study also showed that the range of $\delta^{13}C$ values was greater in males than in females, although this difference was not significant. Therefore, it is possible that males and females eat different foods, resulting in differences in metal accumulation between the sexes. By contrast, the Hg concentration was higher in males than in females (Table 6). Short-tailed shearwaters will accumulate Hg continuously but females will excrete some of this when they lay their single egg, which occurs at around the end of November in south-eastern Australia (Serventy and Curry, 1984). The birds that were used in this

study had died during the summer, almost 1 year after they had bred, which may account for these sex differences.

Juvenile short-tailed shearwaters had higher levels of $\delta^{13}C$ and $\delta^{15}N$ than adults, indicating that these different age groups take different foods. Therefore, the metal concentrations in individuals could reflect both differences in food and the continued accumulation with age.

4.3. Sources of Cd accumulation

Tufted puffin and northern fulmar accumulated high concentrations of Cd. The Cd concentrations at which renal tubular necrosis occurs in seabirds are unclear (Burger, 2008; Stewart et al., 1996), but White and Finley (1978) previously showed that mallard ducks (*Anas platyrhynchos*) with Cd concentrations of >300 mg/kg dry weight in the kidney exhibit this condition. Seabirds have been shown to contain high concentrations of Cd (Hutton, 1981) and some seabird colonies exhibit kidney diseases caused by Cd (Norheim, 1987). Therefore, given the high levels of Cd accumulation observed in the present study, it is possible that even those birds that appeared healthy may have been affected in some way.

Many seabirds change their diet depending on the place (Table 4) and season (Piatt, 2002a, 2002b). Post et al. (2007) found that squid make up a very large proportion of the adult tufted puffin diet in oceanic habitats. In the northern North Pacific, northern fulmars and tufted puffins often feed on squid, and horned puffins sometimes eat squid, while thick-billed murres and short-tailed shearwaters feed mainly on krill and fish (Table 4). Therefore, since squid accumulate high levels of Cd (Stewart et al., 1996; Storelli,

2008), the high Cd concentrations that were found in some species may be related to their squid intake.

4.4. Metal accumulation in seabirds from the Bering Sea

The concentration of Hg was higher in the liver than in the kidneys in northern fulmar (Table 2), short-tailed shearwater, and thick-billed murre, whereas the reverse was true for tufted puffin and horned puffin. It has previously been reported that species that accumulate high levels of Hg have higher concentrations in the liver, whereas species that accumulate low levels of Hg have almost the same levels in the liver and kidneys (Honda et al., 1990), as was the case in the present study. Since the level of accumulation will depend on both the total Hg level and the species-specific demethylation capacity, it is also important to consider the proportion of organic and inorganic Hg in the total Hg alongside differences in the level of accumulation among tissues. Higher levels of Hg in the liver than the kidney would indicate that MeHg is demethylated to produce the inorganic form in the liver (Kim et al., 1996). Only very small amounts of inorganic Hg are absorbed by the intestine, whereas MeHg can be absorbed nearly completely (Wolfe et al., 1998). However, it has been shown that different bird species have different levels of inorganic Hg absorption (Serafin, 1984) and so it follows that their capacity for MeHg absorption will also vary.

The Hg concentrations in the livers and kidneys of seabirds collected in the central Bering Sea in the present study were almost twice as high as those measured in the same species of seabirds collected in 1990 (Honda et al., 1990) (Fig. 4). By contrast, there has been no similar increase in the concentrations of other heavy metals (Cd, Cu, and Zn), suggesting that this increase in Hg concentration is real and may cause poisoning

in various animals in the future. However, the Hg concentrations in birds on Aleutian Island in 2000–2001 were lower than in the present study for northern fulmar (5.44–32.7 vs. 6.82 – 40.3 mg/kg dry weight of liver, respectively) but higher than in the present study for tufted puffin (2.1–4.4 vs. 1.02–1.99 mg/kg dry weight of liver, respectively) (Ricca et al., 2008). Therefore, it is possible that the Hg concentration is not increasing across all species. However, the increased concentration in seabirds that readily accumulate Hg, such as northern fulmar, may have some effect.

4.5. Metal accumulation in seabirds from Teuri Island

Spectacled guillemots eat fish such as small righteye flounders, and crustaceans and other invertebrates, and Uneyama et al. (2007) found that righteye flounders and crustaceans have higher levels of As than other species in Japan. In the present study, one spectacled guillemot had a high renal Cd level, which may have been correlated with the fact that this species mainly feeds on Japanese anchovy (*Engraulis japonicus*) (Ito et al., 2009; Takahashi et al., 2001), which had a tendency to accumulate higher concentrations of Hg than Atka mackerel (*Pleurogrammus monopterygius*) (data not shown). Although benchmark values for nephrotoxicity by Cd in seabirds are unclear (Burger, 2008; Stewart et al., 1996), this high level of Cd indicates the possibility of kidney injury.

Rhinoceros auklet accumulated higher concentrations of Hg than other species on Teuri Island, and these were similar to the levels of Hg accumulation previously detected by Elliott and Scheuhammer (1997) in rhinoceros auklets from Lucy Island and Storm Island on the Canadian Pacific coast (5.3 ± 1.5 vs. 3.6 ± 1.3 and 5.4 ± 1.2 mg/kg dry weight of liver, respectively). By contrast, the Cd levels that were observed in the present study were relatively low compared with those recorded in rhinoceros auklets on

Lucy Island, Storm Island, and Cleland Island (4.1 ± 1.9 vs. 43.5 ± 11.3, 22.9 ± 6.9, and 20.5 ± 6.4 mg/kg dry weight of liver, respectively), indicating that Teuri Island is less polluted by Cd than the Canadian Pacific coast. No previous data have been collected on metal pollution in birds inhabiting Teuri Island, and few data have been collected for rhinoceros auklet and spectacled guillemot from other areas in recent years. Therefore, further research on these species, including resampling around Teuri Island, is required to further our knowledge of metal pollution in seabirds.

Some of the species that were included in this study are migratory birds and so the accumulation of metals would include pollutants from other areas. Rhinoceros auklets migrate from the northern Pacific or other parts of Japan to Teuri Island from March to August, after which they are believed to move to around the Sea of Okhotsk to increase their fish intake for breeding. Similarly, spectacled guillemots migrate from Honshu, the main island of Japan, to Teuri Island from April to November. Therefore, both species might be affected by contamination in these other areas, although given the amounts of feeding they carry out on Teuri Island, their accumulated metals may reflect the level of pollution in northern parts of Hokkaido. Thick-billed murres migrate from the Arctic Ocean to overwinter on Teuri Island and so most of their metal accumulation will reflect the contamination status here. The present study showed that Teuri Island is less polluted than many other areas around the world. However, according to a report of the United Nations Environment Programme (UNEP, 2013), Hg concentrations in arctic marine animals are generally approximately 10–12 times higher now than they were in pre-industrial times. Therefore, given the possibility that Teuri Island will become quite polluted by Hg in the future, continued monitoring of metal pollution is needed here. The results of the present study serve as useful baseline data against for future comparisons.

5. Conclusions

In conclusion, the present study showed that 1) seabird species in the Bering Sea at high trophic levels, such as northern fulmar, accumulate more Hg than those at lower trophic levels; 2) species that feed largely on squid, such as tufted puffin and northern fulmar, readily accumulate Cd; 3) the accumulation pattern of several metals in short-tailed shearwaters depends on the sex and age of the individuals; and 4) the Hg concentration in the central Bering Sea has increased over the past two decades. Together, these findings indicate that the risk of heavy metal accumulation is increasing in this region, which will adversely affect seabirds. This is the first study to collect data on metal accumulation in seabirds on Teuri Island, which will serve as useful baseline data for future comparisons.

Table 1. Sample number, body weight (BW: g) and stable isotope ratio in muscle (δ13C and δ15N, ‰) of the seabirds from the Bering Sea. Different letters indicate significant differences among species (Tukey test, $p < 0.05$)

Species	Number	Age	Sex	BW (g)	δ15N	δ13C
Northern fulmar *F. glacialis*	4	4Ad	1M & 2F & 1U	616.3 ± 56.9 ^{C}	13.0 ± 0.54 ^{A}	-21.0 ± 0.64
Thick-billed murre *U. lomvia*	4	1Ad & 3Ju	1M & 2F & 1U	1066.0 ± 37.1 ^{A}	11.2 ± 0.98 ^{AB}	-21.5 ± 1.86
Short-tailed shearwater *P. tenuirostris*	24	14Ad & 8Ju	9M & 7F & 8U	510.2± 61.3 ^{D}	10.3 ± 0.74 ^{BC}	-22.0 ± 1.27
Tufted puffin *F. cirrhata*	5	1Ad & 3Ju &	4M & 1F	840.8 ± 72.4 ^{B}	9.27 ± 1.01 ^{CD}	-22.7 ± 0.36
Horned puffin *F. corniculata*	3	3Ad	2M & 1F	631.7 ± 78.2 ^{C}	7.02 ± 0.20 ^{D}	-23.4 ± 0.04

Ad: adult, Ju: juvenile, M: male, F: female, U: unidentified

Table 2. Metal and metalloid concentrations in liver and kidney (mg/kg dry weight) of the seabirds from the Bering Sea. Different letters indicate significant differences among species (Tukey test, $p < 0.05$)

Species		Hg	Cd	Cr	Co	Ni	Cu	Zn	As	Pb
Northern fulmar	liver	24.5 ± 12.6 ^{A}	26.7 ± 11.3 ^{A}	0.15 ± 0.09	0.05 ± 0.01	0.06 ± 0.07	16.4 ± 3.60	135.2 ± 25.1 ^{AB}	1.50 ± 1.76	0.06 ± 0.04
F. glacialis	kidney	7.69 ± 4.12 ^{A}	102.7 ± 31.7 ^{AB}	0.32 ± 0.05	0.16 ± 0.04 ^{B}	0.80 ± 1.25 ^{B}	15.3 ± 1.76	148.6 ± 9.49 ^{AB}	1.22 ± 1.06 ^{AB}	0.38 ± 0.27
Thick-billed murre	liver	1.89 ± 1.75 ^{B}	12.7 ± 4.85 ^{B}	0.47 ± 0.24	0.09 ± 0.02	0.84 ± 0.76	18.0 ± 2.01	99.9 ± 21.0 ^{AB}	0.34 ± 0.45	0.02 ± 0.01
U. lomvia	kidney	1.21 ± 0.88 ^{B}	27.4 ± 13.4 ^{AB}	0.45 ± 0.21	0.12 ± 0.03 ^{B}	2.42 ± 3.53 ^{B}	17.1 ± 1.52	103.4 ± 9.46 ^{BC}	0.33 ± 0.38 ^{B}	0.11 ± 0.06
Short-tailed shearwater	liver	1.33 ± 0.66 ^{B}	6.06 ± 4.06 ^{B}	0.28 ± 0.32	0.11 ± 0.08	0.53 ± 0.74	21.5 ± 10.7	105.2 ± 32.8 ^{B}	0.69 ± 0.58	0.08 ± 0.08
P. tenuirostris	kidney	0.83 ± 0.40 ^{B}	43.4 ± 49.4 ^{B}	0.39 ± 0.21	0.18 ± 0.11 ^{B}	2.40 ± 3.79 ^{B}	22.9 ± 13.8	102.7 ± 20.5 ^{C}	0.60 ± 0.45 ^{B}	0.26 ± 0.35
Tufted puffin	liver	1.44 ± 0.33 ^{B}	36.0 ± 9.31 ^{A}	0.10 ± 0.03	0.13 ± 0.04	1.48 ± 1.03	24.4 ± 4.71	156.1 ± 21.1 ^{A}	1.80 ± 0.88	0.03 ± 0.01
F. cirrhata	kidney	1.63 ± 0.41 ^{B}	114.4 ± 50.1 ^{A}	0.42 ± 0.12	0.53 ± 0.37 ^{A}	15.5 ± 6.40 ^{A}	22.9 ± 10.8	153.2 ± 40.9 ^{A}	1.53 ± 0.35 ^{A}	0.04 ± 0.03
Horned puffin	liver	1.63 ± 0.22 ^{B}	10.5 ± 5.30 ^{B}	0.45 ± 0.27	0.10 ± 0.02	0.79 ± 0.54	18.0 ± 2.62	103.6 ± 16.6 ^{AB}	0.68 ± 0.85	0.03 ± 0.02
F. corniculata	kidney	2.50 ± 0.16 ^{B}	52.8 ± 36.4 ^{AB}	0.31 ± 0.10	0.28 ± 0.13 ^{AB}	3.76 ± 5.27 ^{B}	18.8 ± 4.09	120.9 ± 20.7 ^{ABC}	0.54 ± 0.55 ^{AB}	0.17 ± 0.17

Table 3. Sample number, metal and metalloid concentrations in the liver of birds (mg/kg dry weight). Different letters indicate significant differences among species (Tukey's HSD test, $p < 0.05$). Thick-billed Murre was excluded from statistics because of their sample size ($n = 2$).

Species	Number	Hg	Cd	Cr	Co	Ni	Cu	Zn	As	Pb
Slaty-backed Gull *Larus schistisagus*	15	6.04 ± 3.51	4.52 ± 2.42	0.07 ± 0.06	0.08 ± 0.04	0.90 ± 1.46	10.3 ± 1.78	81.0 ± 26.1	0.19 ± 0.12	0.08 ± 0.10
Rhinoceros Auklet *Cerorhinca monocerata*	7	5.25 ± 1.50	4.06 ± 1.89	0.12 ± 0.06	0.09 ± 0.02	0.30 ± 0.24	19.3 ± 3.63	81.7 ± 16.6	2.44 ± 1.17	0.14 ± 0.10
Spectacled Guillemot *Cepphus carbo*	6	4.14 ± 1.52	7.01 ± 7.80	0.10 ± 0.02	0.07 ± 0.01	0.25 ± 0.06	19.8 ± 2.92	61.2 ± 7.94	15.2 ± 6.74	0.11 ± 0.14
Thick-billed Murre *Uria lomvia*	2	2.63 ± 1.65	3.47 ± 1.89	0.12 ± 0.04	0.06 ± 0.01	0.24 ± 0.12	15.9 ± 1.18	71.4 ± 8.74	2.72 ± 1.55	0.06 ± 0.02

Table 4. Prey (crustacean, fish and squid) composition (% mass or volume) in the stomach contents in seabirds collected in the Bering Sea and Aleutians.

Species	Area	Fish	Squids	Crustaceans	Refs
Northern fulmar	Bering Sea	73	21	6	(Hunt Jr et al. 1981)
F. glacialis	Gulf of Alaska	3	96	1	(DeGange and Sanger 1986) cited in (Hunt et al. 2005)
Thick-billed murre	Bering Sea	95	1	4	(Hunt Jr et al. 1981)
U. lomvia	Bering Sea	6	<1	82	(Ogi and Hamanaka 1982)
	Okhotsk	82	<1	11	(Ogi and Tsujita 1978)
	Gulf of Alaska	16	74	10	(DeGange and Sanger 1986) cited in (Hunt et al. 2005)
	Chukchi Sea	100	0	0	(Piatt et al. 1991)
Short-tailed shearwater	Bering Sea	0-67	0	33-100	(Hunt et al. 2002)
P. tenuirostris	Bering Sea	8-40	0-30	36-82	(Toge et al. 2011)
	Northern North Pacific	12	1	87	(Ogi et al. 1980)
	Gulf of Alaska	24	2	73	(DeGange and Sanger 1986) cited in (Hunt et al. 2005)
Tufted puffin	Bering Sea	81	2	3	(Hunt Jr et al. 1981)
F. cirrhata	Bering Sea	33.6	60.5	0	(Tanaka 1989)
	Pribilofs	99.3	0	0.7	(Johnson 1985)
	Aleutians	41.2	41.5	17	(Tanaka 1989)
	Gulf of Alaska	81	8	11	(DeGange and Sanger 1986) cited in (Hunt et al. 2005)
Species	Area	Fish	Squids	Crustaceans	Refs
Horned puffin	Bering Sea	80	1	11	(Hunt Jr et al. 1981)
F. corniculata	Pribilofs	52.1	47.5	0.4	(Johnson 1985)
	Aleutians	62.4	33.4	0.8	(SPRINGER et al. 1996) cited in (Piatt 2002a)
	Gulf of Alaska	98	1	1	(DeGange and Sanger 1986) cited in (Hunt et al. 2005)

Table 5. Pairwise correlation coefficients *r* (*r* > 0.5 or *r* < - 0.5) for body weight, each metal and metalloid in the liver and stable isotope ratio in muscle of various species from the Bering Sea and Teuri Island (*p* < 0.05).

	r
$\delta^{13}C$ - d15N	0.84
$\delta^{15}N$ - Hg	0.69
$\delta^{13}C$ - Hg	0.56
As - Cu	0.56
$\delta^{13}C$ - BW	0.55
Zn - Cd	0.54
$\delta^{15}N$ - Cr	-0.54
Cr - BW	-0.54
Cu - BW	-0.58
$\delta^{13}C$ - Cr	-0.67

Table 6. Gender and age differences in body weight (g), metal concentration (mg/kg, dry weight in liver), and stable isotope ratio (δ13C and δ15N, ‰) in short-tailed shearwater.

Given are arithmetic means ± SD.

	Male (n = 9)	Female (n = 7)	Kruskal-Wallis X^2 (p)
Body weight	476 ± 62	541 ± 28	3.85 (0.05)
Hg	1.71 ± 0.74	0.88 ± 0.36	5.18 (0.02)
Cd	3.06 ± 1.97	9.55 ± 4.14	8.47 (0.004)
Pb	0.05 ± 0.02	0.09 ± 0.03	4.29 (0.04)

	Adult (n = 14)	Juvenile (n = 8)	Kruskal-Wallis X^2 (p)
Body weight	497 ± 55	505 ± 47	0.09 (0.76)
Cr	0.37 ± 0.38	0.15 ± 0.15	8.44 (0.004)
Co	0.11 ± 0.10	0.13 ± 0.04	4.11 (0.04)
Cu	17.7 ± 5.53	29.4 ± 14.1	9.43 (0.002)
$\delta^{13}C$	10.1 ± 0.66	10.8 ± 0.60	6.43 (0.01)
$\delta^{15}N$	-22.4 ± 1.18	-21.0 ± 0.92	5.36 (0.02)

Fig. 1. Map of the Bering Sea.
The dotted line indicates the sampling area.

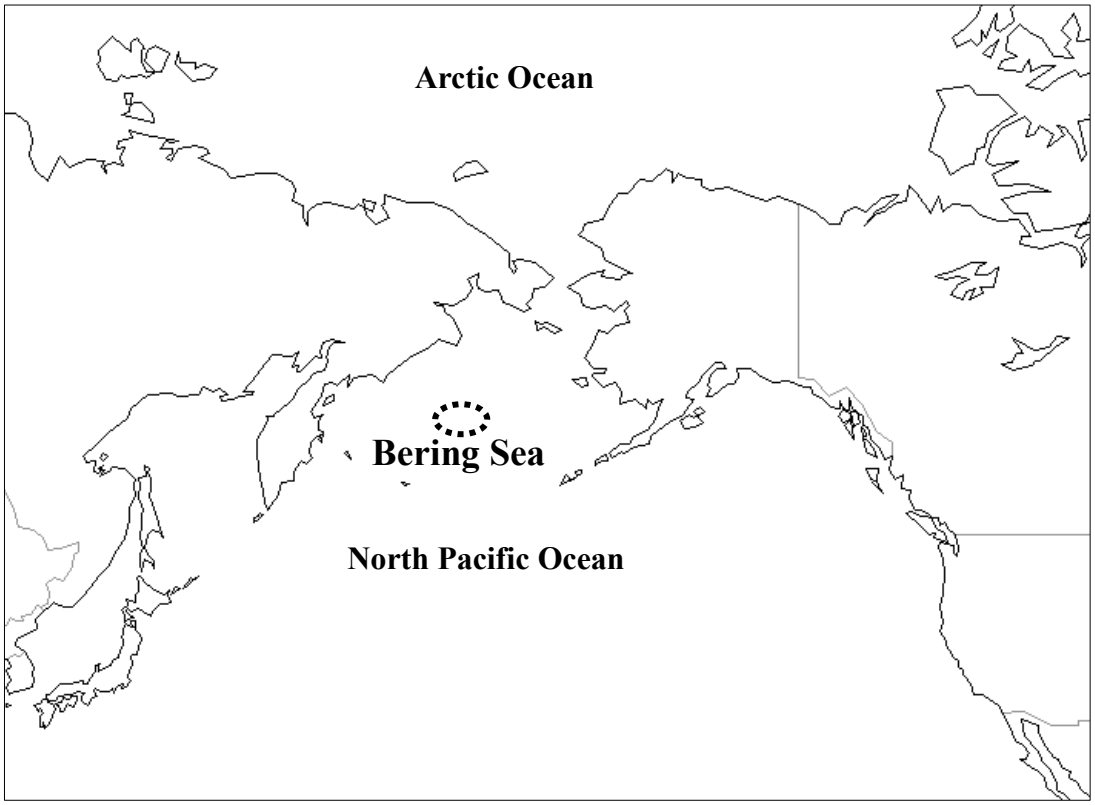


Fig. 2. Map of the Teuri Island.

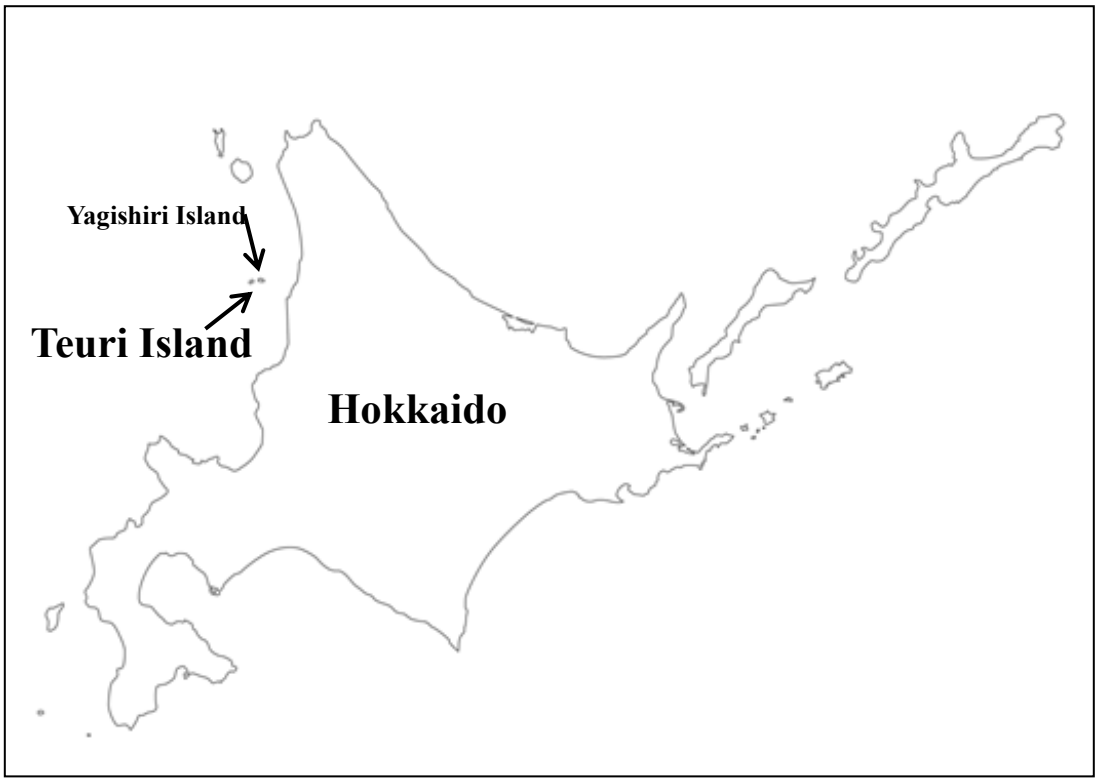


Fig. 3. Principal component analysis (PCA) of body weight (BW), metal and metalloid concentrations (mg/kg dry weight) in liver, and stable isotope ratio ($\delta^{15}N$, $\delta^{13}C$) in muscle.

Specimens; northern fulmar (purple), thick-billed murre (blue), short-tailed shearwater (orange), tufted puffin (pink), horned puffin (green), slaty-backed gull (grey), rhinoceros auklet (yellow green), spectacled guillemot (red). Circles indicate seabirds from the Bering Sea and triangles mean the seabirds from Teuri Island.

The value of component 1 plus component 2 were low (43.2%).

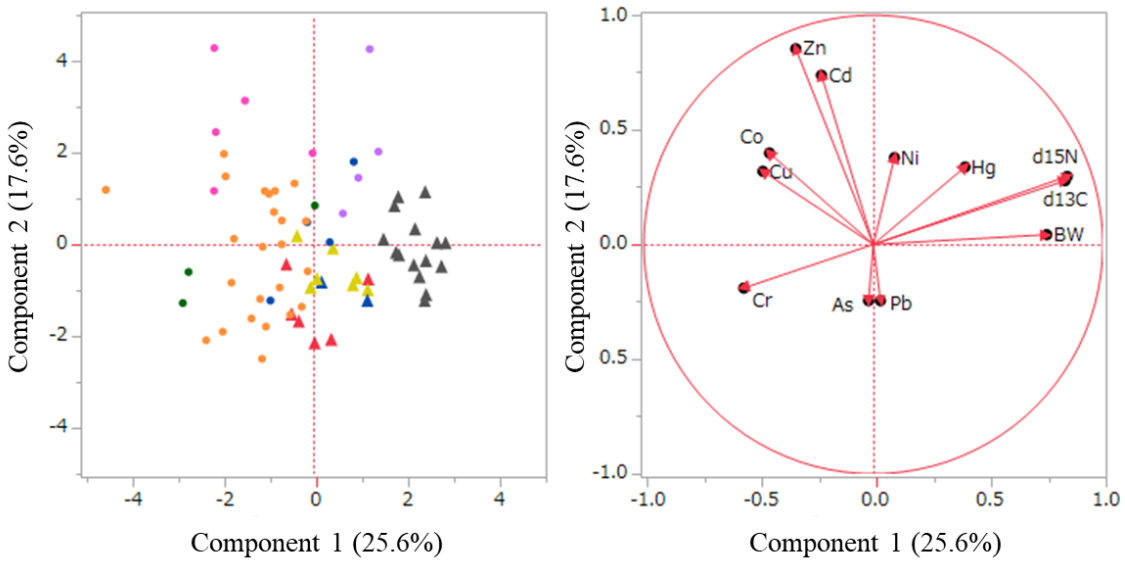
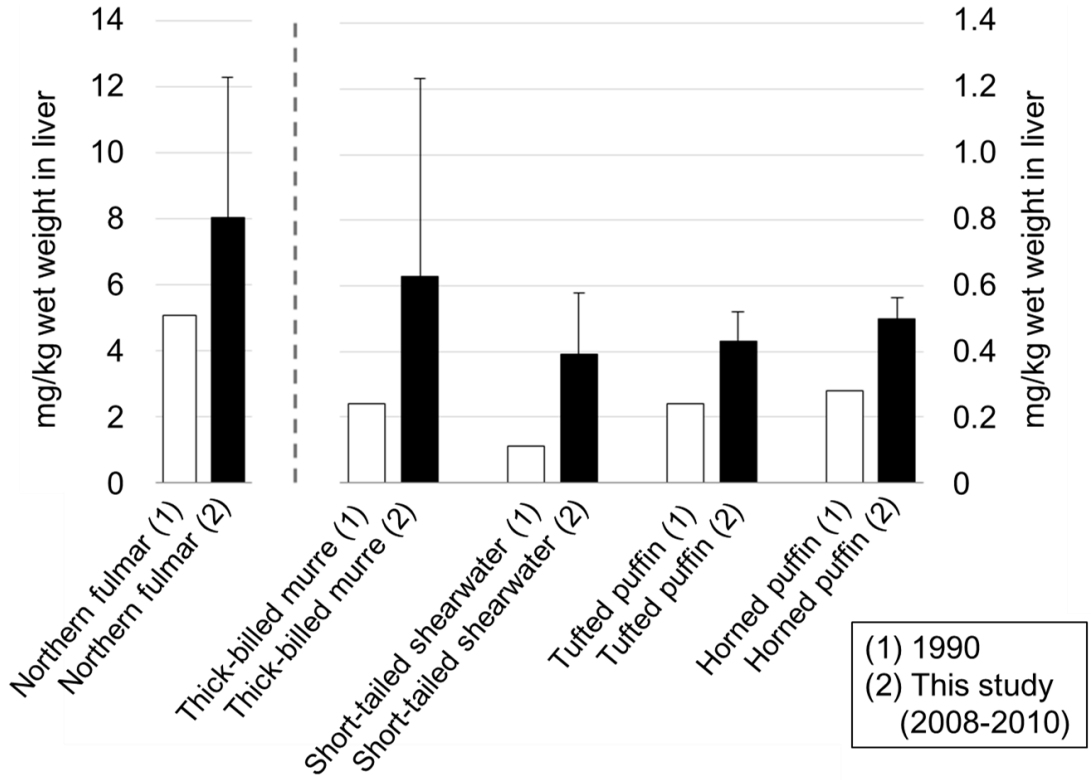


Fig. 4. Comparison of Hg levels in the livers of seabirds in the Bering Sea during the periods (1) 1982 – 1985 (Honda et al. 1990) and (2) 2008 – 2010 (this study) (mean, mg/kg wet weight).



References

Alleva, E., Francia, N., Pandolfi, M., De Marinis, A.M., Chiarotti, F., Santucci, D., 2006. Organochlorine and heavy-metal contaminants in wild mammals and birds of Urbino-Pesaro province, Italy: an analytic overview for potential bioindicators. Arch. Environ. Contam. Toxicol. 51, 123–134.

Atwell, L., Hobson, K.A., Welch, H.E., 1998. Biomagnification and bioaccumulation of mercury in an arctic marine food web: insights from stable nitrogen isotope analysis. Can. J. Fish. Aquat. Sci. 55, 1114–1121.

Blevin, P., Carravieri, A., Jaeger, A., Chastel, O., Bustamante, P., Cherel, Y., 2013. Wide range of mercury contamination in chicks of southern ocean seabirds. PLoS One 8, e54508. doi:10.1371/journal.pone.0054508

Bryan Jr., A.L., Brant, H., Jagoe, C., Romanek, C., Brisbin Jr., I.L., 2012. Mercury Concentrations in Nestling Wading Birds Relative to Diet in the Southeastern United States: A Stable Isotope Analysis. Arch. Environ. Contam. Toxicol. 63, 144–152. doi:10.1007/s00244-011-9745-0

Burger, J., 2008. Assessment and management of risk to wildlife from cadmium. Sci. Total Environ. 389, 37–45. doi:http://dx.doi.org/10.1016/j.scitotenv.2007.08.037

Burger, J., 1995. Heavy metal and selenium levels in feathers of herring gulls (*Larus argentatus*): differences due to year, gender, and age at Captree, Long Island. Environ. Monit. Assess. 38, 37–50.

Elliott, J.E., Scheuhammer, A.M., 1997. Heavy metal and metallothionein concentrations in seabirds from the Pacific coast of Canada. Mar. Pollut. Bull. 34, 794–801. doi:http://dx.doi.org/10.1016/S0025-326X(97)00034-9

Elliott, J.E., Scheuhammer, A.M., Leighton, F.A., Pearce, P.A., 1992. Heavy metal and metallothionein concentrations in Atlantic Canadian seabirds. Arch. Environ. Contam. Toxicol. 22, 63–73. doi:10.1007/BF00213303

Fitzgerald, W.F., Lamborg, C.H., Hammerschmidt, C.R., 2007. Marine biogeochemical cycling of mercury. Chem. Rev. 107, 641–662.

Honda, K., Marcovecchio, J., Kan, S., Tatsukawa, R., Ogi, H., 1990. Metal concentrations in pelagic seabirds from the North Pacific Ocean. Arch. Environ. Contam. Toxicol. 19, 704–711. doi:10.1007/BF01183988

Hutton, M., 1981. Accumulation of heavy metals and selenium in three seabird species from the United Kingdom. Environ. Pollut. Ser. A, Ecol. Biol. 26, 129–145. doi:http://dx.doi.org/10.1016/0143-1471(81)90043-X

Ito, M., Minami, H., Tanaka, Y., Watanuki, Y., 2009. Seasonal and inter-annual oceanographic changes induce diet switching in a piscivorous seabird. Mar. Ecol. Prog. Ser. 393, 273–284.

Kim, E.Y., Murakami, T., Saeki, K., Tatsukawa, R., 1996. Mercury levels and its chemical form in tissues and organs of seabirds. Arch. Environ. Contam. Toxicol. 30, 259–266. doi:10.1007/BF00215806

Larison, J.R., Likens, G.E., Fitzpatrick, J.W., Crock, J.G., 2000. Cadmium toxicity among wildlife in the Colorado Rocky Mountains. Nature 406, 181–183.

Lucia, M., André, J.-M., Gontier, K., Diot, N., Veiga, J., Davail, S., 2010. Trace Element Concentrations (Mercury, Cadmium, Copper, Zinc, Lead, Aluminium, Nickel, Arsenic, and Selenium) in Some Aquatic Birds of the Southwest Atlantic Coast of France. Arch. Environ. Contam. Toxicol. 58, 844–853. doi:10.1007/s00244-009-9393-9

Mason, R.P., Fitzgerald, W.F., Morel, F.M.M., 1994. The biogeochemical cycling of elemental mercury: anthropogenic influences. Geochim. Cosmochim. Acta 58, 3191–3198.

Minagawa, M., Wada, E., 1984. Stepwise enrichment of 15 N along food chains: further evidence and the relation between δ 15 N and animal age. Geochim. Cosmochim. Acta 48, 1135–1140.

Nakayama, S.M., Ikenaka, Y., Muzandu, K., Choongo, K., Yabe, J., Muroya, T., Ijiri, S., Minagawa, M., Umemura, T., Ishizuka, M., 2013. Geographic Information System-Based Source Estimation of Copper Pollution in Lake Itezhi-tezhi and Metal-Accumulation Profiles in Oreochromis spp. from Both Field and Laboratory Studies. Arch. Environ. Contam. Toxicol. 64, 119–129. doi:10.1007/s00244-012-9802-3

Norheim, G., 1987. Levels and interactions of heavy metals in sea birds from Svalbard and the Antarctic. Environ. Pollut. 47, 83–94. doi:http://dx.doi.org/10.1016/0269-7491(87)90039-X

Overland, J.E., Stabeno, P.J., 2004. Is the climate of the Bering Sea warming and affecting the ecosystem? Eos, Trans. Am. Geophys. Union 85, 309–312. doi:10.1029/2004EO330001

Piatt, J.F., 2002a. Horned puffin (*Fratercula corniculata*). birds North Am. 611, 1–27.

Piatt, J.F., 2002b. Tufted puffin (*Fratercula cirrhata*). Birds North Am. 708, 1–31.

Pirrone, N., Cinnirella, S., Feng, X., Finkelman, R.B., Friedli, H.R., Leaner, J., Mason, R., Mukherjee, A.B., Stracher, G.B., Streets, D.G., 2010. Global mercury emissions to the atmosphere from anthropogenic and natural sources. Atmos. Chem. Phys. 10, 5951–5964.

Post, D.M., Layman, C.A., Arrington, D.A., Takimoto, G., Quattrochi, J., Montana, C.G., 2007. Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analyses. Oecologia 152, 179–189.

Ricca, M.A., Keith Miles, A., Anthony, R.G., 2008. Sources of organochlorine contaminants and mercury in seabirds from the Aleutian archipelago of Alaska: inferences from spatial and trophic variation. Sci. Total Environ. 406, 308–23. doi:10.1016/j.scitotenv.2008.06.030

Robinson, S.A., Forbes, M.R., Hebert, C.E., Scheuhammer, A.M., 2011. Evidence for sex differences in mercury dynamics in double-crested cormorants. Environ. Sci. Technol. 45, 1213–1218.

Scheuhammer, A.M., 1987. The chronic toxicity of aluminium, cadmium, mercury, and lead in birds: A review. Environ. Pollut. 46, 263–295. doi:http://dx.doi.org/10.1016/0269-7491(87)90173-4

Serafin, J.A., 1984. Avian species differences in the intestinal absorption of xenobiotics (PCB, dieldrin, Hg2+). Comp. Biochem. Physiol. Part C Comp. Pharmacol. 78, 491–496. doi:http://dx.doi.org/10.1016/0742-8413(84)90120-8

Serventy, D.L., Curry, P.J., 1984. Observations on colony size, breeding success, recruitment and inter-colony dispersal in a Tasmanian colony of Short-tailed Shearwaters Puffinus tenuirostris over a 30-year peroid. Emu 84, 71–79.

Stewart, F.M., Furness, R.W., Monteiro, L.R., 1996. Relationships between heavy metal and metallothionein concentrations in lesser black-backed gulls, *Larus fuscus*, and Cory's shearwater, *Calonectris diomedea*. Arch. Environ. Contam. Toxicol. 30, 299–305. doi:10.1007/BF00212287

Storelli, M.M., 2008. Potential human health risks from metals (Hg, Cd, and Pb) and polychlorinated biphenyls (PCBs) via seafood consumption: Estimation of target hazard quotients (THQs) and toxic equivalents (TEQs). Food Chem. Toxicol. 46, 2782–2788. doi:http://dx.doi.org/10.1016/j.fct.2008.05.011

Takahashi, A., Kuroki, M., Niizuma, Y., Kato, A., Saitoh, S., Watanuki, Y., 2001. Importance of the Japanese anchovy (*Engraulis japonicus*) to breeding rhinoceros auklets (*Cerorhinca monocerata*) on Teuri Island, Sea of Japan. Mar. Biol. 139, 361–371.

Uneyama, C., Toda, M., Yamamoto, M., Morikawa, K., 2007. Arsenic in various foods: cumulative data. Food Addit Contam 24, 447–534. doi:10.1080/02652030601053121

United Nations Environment Programme (UNEP). 2013. Global Mercury Assessment 2013: Sources, Emissions, Releases and Environmental Transport. UNEP Chemicals Branch, Geneva

Watanuki, Y., Yamamoto, T., Yamashita, A., Ishii, C., Ikenaka, Y., Nakayama, S.M.M., Ishizuka, M., Suzuki, Y., Niizuma, Y., Meathrel, C.E., 2015. Mercury concentrations in primary feathers reflect pollutant exposure in discrete non-breeding grounds used by Short-tailed Shearwaters. J. Ornithol. 1–4.

White, D.H., Finley, M.T., 1978. Uptake and retention of dietary cadmium in mallard ducks. Environ. Res. 17, 53–59.

Wolfe, M.F., Schwarzbach, S., Sulaiman, R.A., 1998. Effects of mercury on wildlife: A comprehensive review. Environ. Toxicol. Chem. 17, 146–160. doi:10.1002/etc.5620170203

Yabe, J., Nakayama, S.M.M., Ikenaka, Y., Muzandu, K., Choongo, K., Mainda, G., Kabeta, M., Ishizuka, M., Umemura, T., 2013. Metal distribution in tissues of free-range chickens near a lead–zinc mine in Kabwe, Zambia. Environ. Toxicol. Chem. 32, 189–192. doi:10.1002/etc.2029

Zhang, D., Gao, J., Zhang, K., Liu, X., Li, J., 2012. Effects of Chronic Cadmium Poisoning on Zn, Cu, Fe, Ca, and Metallothionein in Liver and Kidney of Rats. Biol. Trace Elem. Res. 149, 57–63. doi:10.1007/s12011-012-9394-9

CHAPTER 2

Lead exposure in raptors and source identification using lead isotope ratios

Abstract

Lead (Pb) poisoning is widespread among raptors and waterbirds. In Japan, fragments of Pb ammunition are still found in endangered eagles although more than 10 years have passed since legislation regarding use of Pb ammunition was introduced. This study was performed to investigate Pb exposure in raptors from various locations in Japan. We measured hepatic and renal Pb concentrations and hepatic Pb isotope ratios of Steller's sea eagles (*Haliaeetus pelagicus*), white-tailed sea eagles (*Haliaeetus albicilla*), golden eagles (*Aquila chrysaetos*), and 13 other species (total 177 individuals) that were found dead, as well as blood samples from three eagles found in a weakened state during 1993 – 2015 from Hokkaido (northern part), Honshu (the main island), and Shikoku (a southern island) of Japan. In the present study in Hokkaido, one quarter of the sea eagles showed a high Pb concentration, suggesting exposure to abnormally high Pb levels and Pb poisoning. Pb isotope ratios indicated that endangered Steller's sea eagle and white-tailed sea eagle were poisoned by Pb ammunition that was used illegally in Hokkaido. In other areas of Japan, both surveillance and regulations were less extensive than in Hokkaido, but Pb poisoning in raptors was also noted. Therefore, Pb poisoning is still a serious problem in raptors in various areas of Japan due to accidental ingestion of materials containing Pb, especially Pb ammunition.

Keywords

Pb exposure, Raptor, Pb ammunition, Pb isotope ratios, Japan

Highlights

- In Hokkaido, one quarter of sea eagles showed a high Pb concentration, suggesting exposure to abnormally high Pb levels.
- Pb isotope ratios indicated that sea eagles in Hokkaido were poisoned by Pb ammunition that were likely used illegally.
- Pb poisoning is still a serious problem in raptors in various areas of Japan due to accidental ingestion of Pb ammunition.

1. Introduction

Lead (Pb) poisoning has been widespread among raptors and waterbirds (Fisher et al., 2006; Kendall et al., 1996; Kim et al., 1999; Kurosawa, 2000; Saito, 2009) since the 1870s (Rattner, 2009). Raptors mainly ingest fragments of Pb rifle bullets or shot pellets when consuming animals killed by hunters. Pb is dissolved rapidly in the stomach of raptors by the low-pH gastric acid and subsequently absorbed (Saito, 2009), exposing raptors to high Pb concentrations. Waterbirds also tend to accidentally ingest Pb from shot pellets or fishing sinkers when they swallow pebbles as gastroliths (Martinez-Haro et al., 2011; Pain et al., 2007).

Pb exposure causes neurological dysfunction, hematopoietic system dysfunction, immune suppression, reproductive impairment, and with accumulation of Pb at very high levels, it eventually leads to death. Even at low levels, Pb exposure deprives birds of bodily strength (Haig et al., 2014; Kendall et al., 1996; Saito, 2009). Poor health condition increases susceptibility to illness, making it difficult to accomplish migration. Pb exposure at non-lethal levels has been linked to other causes of death, such as traffic accidents (Saito, 2009). A significant positive association has been found between collision/electrocution/trauma and Pb contamination in raptors (Berny et al., 2015), and Pb exposure effects on reproduction in an avian model (Vallverdú-Coll et al., 2016). Therefore, Pb poisoning may be one of the causes for the decline in raptor populations mainly due to the death of raptors, and also poisoning inhibits their breeding activities and success.

Various types of wildlife—including endangered species—inhabit the six national parks in, Hokkaido, the northernmost island of Japan (Fig. S1 in Supplementary data). The world's Steller's sea eagle (*Haliaeetus pelagicus*) population is only 4600 –

5100, and white-tailed sea eagle (*Haliaeetus albicilla*) population is approximately 20300 – 39600 (IUCN, 2015). In Japan, 1400 – 1700 of Steller's sea eagles and 700 – 900 of white-tailed sea eagles migrate to Hokkaido to spend the winter (Ministry of the Environment, Japan, 2016, *in Japanese*). Both types of sea eagle are protected with the "Act on Conservation of Endangered Species of Wild Fauna and Flora" in Japan from 1993 (Ministry of the Environment, Japan).

In Hokkaido, the population of sika deer has been increasing, and the government encourages people to control the number of deer. Hunters have used Pb rifle bullets to hunt sika deer, as a consequence raptors have been exposed to Pb by consuming deer carcasses containing Pb fragments (Iwata et al., 2000; Kim et al., 1999; Kurosawa, 2000). It is reported that Pb poisoning accounted for 79% of all deaths of these types of eagles in winter 1998 – 1999 (26 of 33 cases) (Saito, 2009). Therefore, Pb rifle bullets and shot pellets for hunting sika deer have been prohibited since 2000 and 2001, respectively. After the regulation, hunters were required to use much less toxic material, such as copper (Cu) instead of Pb ammunition. However, the incidence of Pb poisoning in the total number of raptor deaths remained high (69% in winter 2001 – 2002, 11 of 16 cases reported by Wildlife Preservation Bureau of Hokkaido Corporation, and Saito (2009)). In 2004, an extended ban was implemented in Hokkaido, which prohibited the use of any type of Pb containing ammunition for hunting of large-sized animal species.

In other areas of Japan, such as Honshu (the main island) and Shikoku (a southern island) (Fig. S1), there are few regulations regarding the use of Pb containing ammunition and the current situation of Pb poisoning is unknown. Moreover, Pb ammunition is still used in these areas. The golden eagle (*Aquila chrysaetos*), which

inhabits Honshu, is an endangered species with a population of only 500 in Japan (Kyodo, 2015).

Pb poisoning in wild birds is still being reported worldwide (Haig et al., 2014; Langner et al., 2015; Madry et al., 2015), even though some countries, such as the USA and Denmark, have introduced regulations to curb the incidence of such poisoning (Finkelstein et al., 2014; Mateo, 2009). Therefore, it is necessary to accurately determine the occurrence of Pb poisoning in raptors to develop appropriate regulations for their conservation.

There are many sources of Pb poisoning, and Pb isotope ratios (Pb isotope ratios; $^{207}Pb/^{206}Pb$, and $^{208}Pb/^{206}Pb$ values) are useful for identifying possible exposure sources (Church et al., 2006; Komárek et al., 2008; Pain et al., 2007; Scheuhammer and Templeton, 1998). There are four stable isotopes of Pb: ^{204}Pb, ^{206}Pb, ^{207}Pb, and ^{208}Pb. The combination of $^{208}Pb/^{206}Pb$ and $^{207}Pb/^{206}Pb$ ratios differs depending on the original source of Pb.

This study was performed to investigate the occurrence of Pb exposure in raptors from various locations, such as Hokkaido, Honshu and Shikoku in Japan and to identify the sources of Pb by using stable isotope ratios.

2. Materials and Methods

2.1. Sampling

Samples of birds that died in nature, in medical centers for wild birds, and carcasses kept in museums or universities were collected from various areas in Japan for analysis of Pb concentration and Pb isotope ratios. The liver and kidney samples of white-tailed sea eagle ($n = 51$), Steller's sea eagle ($n = 47$), Blakiston's fish owl (*Ketupa blakistoni*) ($n = 13$), mountain hawk eagle (*Spizaetus nipalensis*) ($n = 7$), northern goshawk (*Accipiter gentilis*) ($n = 6$), sparrow hawk (*Accipiter nisus*) ($n = 2$), peregrine falcon (*Falco peregrinus*) ($n = 1$), and black kite (*Milvus migrans*) ($n = 1$), as well as blood samples from a mountain hawk eagle ($n = 1$) and a white-tailed sea eagle ($n = 1$), were collected by the Institute for Raptor Biomedicine Japan and Shiretoko Museum in the eastern part of Hokkaido, Japan, from 1998 to 2015. From Honshu and Shikoku, liver samples of golden eagle ($n = 13$), northern goshawk ($n = 9$), black kite ($n = 9$), ural owl (*Strix uralensis*) ($n = 4$), sparrow hawk ($n = 2$), brown hawk owl (*Ninox scutulata*) ($n = 2$), mountain hawk eagle ($n = 1$), peregrine falcon ($n = 1$), osprey (*Pandion haliaetus*) ($n = 1$), gray-faced buzzard (*Butastur indicus*) ($n = 1$), Japanese sparrow hawk (*Accipiter gularis*) ($n = 1$), common kestrel (*Falco tinnunculus*) ($n = 1$), and Sunda scops owl (*Otus lempiji*) ($n = 1$), as well as a blood sample from a golden eagle ($n = 1$), were collected by the Environmental Specimen Bank (*es*-BANK) of Ehime University, Tochigi Prefectural Museum, and the Institute for Raptor Biomedicine Japan from 1993 to 2015. Blood samples were collected from three eagles (mountain hawk eagle, and white-tailed sea eagle from Hokkaido, and golden eagle from Honshu) that were found in a weakened state and were treated at the animal hospital. Samples were transported to the Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan. All samples were

preserved at –20°C until analysis. The age of raptors was estimated by the morphological characteristics, such as the development of the gonad and the feather, and the color of their feather and the iris. The condition of their molting was also determined to estimate their age.

As Pb ammunition; three shot pellets (one is from a hunter, and the other two are from the carcasses of birds), three rifle bullets (one is silver chip from the hunter, and the others are unknown from the stomach of raptors), three slugs (one is produced by Federal, another one is unknown that was found in the stomach of raptor, and the other is from the ground), and one air gun bullet (from the ground) and one sinker (from a fisherman) were also collected to compare Pb isotope ratios with the raptor tissues.

2.2. Pb concentration and Pb stable isotope analysis

Pb concentrations were analyzed according to the method of Yabe et al. (2015). Samples of 100–300 mg of soft tissues were used for the analysis. Subsequently, samples were digested with 5 mL of 30% nitric acid (Kanto Chemical Corporation, Tokyo, Japan) and 1 mL of 30% hydrogen peroxide (Kanto Chemical Corporation) in a microwave digestion system (Speedwave Two; Berghof, Eningen, Germany), after which the volume was brought to 10 mL with 2% nitric acid. Digestion was performed under the following conditions: 180°C for 15 minutes, 200°C for 20 minutes, and 100°C for 20 minutes. Concentration and isotope ratios of Pb were measured with an inductively coupled plasma–mass spectrometer (ICP-MS) (7700 series; Agilent Technology, Tokyo, Japan). The instrument was calibrated using ICP-MS Calibration Standards (Agilent Technology) to establish standard curves before analysis. Standard solutions (0, 10, 50, 100, 250, 500 μg/L) were prepared with 2% nitric acid and the R^2 value of the linear regression line was

0.998. All chemicals and standard stock solutions were of analytical reagent grade (Wako Pure Chemicals Industries, Osaka, Japan). Water was distilled and deionized (Milli-Q; Merck Millipore, Billerica, MA). Analytical quality control was performed using DOLT-4 (dogfish liver) and DORM-3 (fish protein) certified reference material (National Research Council of Canada, Ottawa, Canada). Replicate analysis of these reference materials showed good recoveries (95% – 105%). The limit of detection for Pb was 0.01 μg/kg. For the analysis of Pb concentration, Thallium (^{205}Tl) was used as internal standard, but not for the isotope ratio analyses.

Analysis of Pb isotope ratios was performed according to the method of Nakata et al. (2015). Dissolved samples were diluted to Pb concentration < 25 μg/L with 2% nitric acid. NIST SRM 981 (National Institute of Standards and Technology, Gaithersburg, MD) was used as a standard reference material for the external standardization of Pb isotopes. Detailed analytical conditions are shown in Table S1. The relative standard deviation (RSD) of the ratios was found to be < 0.5% for both ^{207}Pb/^{206}Pb and ^{208}Pb/^{207}Pb. Samples where the RSD value exceeded 0.5% were excluded from the analysis. Standard solutions were measured every 10 samples to correct calibration.

2.3. Assessment of Pb exposure

Various thresholds for Pb toxicity in birds have been reported in the literature (Fisher et al., 2006; Kendall et al., 1996; Kim et al., 1999; Kurosawa, 2000; Saito, 2009). Background level of Pb in the liver of avian is generally < 2 mg/kg wet weight (6 – 7 mg/kg dry weight) or < 1 mg/kg wet weight (3 mg/kg dry weight). The level of > 6 mg/kg dry weight indicates abnormally high exposure to Pb and > 20 mg/kg dry weight indicates acute exposure and absorption, resulting in Pb poisoning (Pain et al., 1995; Pain and

Amiardtriquet, 1993). The categories used in Japan are as follows; hepatic Pb concentration in wet weight: < 0.2 mg/kg, normal range; 0.2 – 2 mg/kg, high level of Pb exposure; and > 2 mg/kg, Pb poisoning. In the blood, Pb concentration in raptors by wet weight is used: < 0.1 mg/kg, normal range; 0.1 – 0.6 mg/kg, high level of Pb exposure; and > 0.6 mg/kg, Pb poisoning (Saito, 2009).

2.4. Statistics

For comparison of Pb concentrations among species, sexes, and ages of Steller's sea eagles and white-tailed sea eagles, data were analyzed using the Mann–Whitney U test (for species, and sexes) or Steel-Dwass test (for ages) with a significance level at $p < 0.05$. Statistical analyses were performed in JMP Pro 11 (SAS Institute, Cary, NC).

3. Results

3.1. Pb concentrations in the liver, kidney, and blood samples of raptors from Hokkaido

Table 1 shows the median hepatic Pb concentrations in the studied raptors from Hokkaido. The results indicated that Pb accumulation in 42% of Steller's sea eagles (18 of 43 cases) and 24% of white-tailed sea eagles (12 of 50 cases) from Hokkaido exceeded the level of Pb poisoning (> 2 mg/kg wet weight in liver). They were collected after the regulation was introduced in 2004, which prohibited the use of any type of Pb ammunition for hunting of large-sized animal species. The Steller's sea eagle had a higher ratio of Pb poisoning than the white-tailed sea eagle, although their Pb levels were not significantly different. Data regarding age, sex, and Pb concentrations (liver, kidney, and blood) are shown in Table S2. In these raptors, renal Pb levels were also high. Blood samples collected after the regulation from one mountain hawk eagle and one white-tailed sea eagle also showed high Pb concentrations (0.38 and 0.16 mg/kg wet weight, respectively). The hepatic Pb levels in the present study were comparable to previous data obtained from 1995 to 1998 (Table S3).

One Steller's sea eagle that died in 2013 was examined by postmortem radiography (Fig. 1), and the slightly large and pointed fragment that indicated a rifle bullet was found in the stomach. Measurements of metal concentrations showed that the bullet fragment was almost entirely (> 90%) composed of Pb. Hepatic Pb level (36.3 mg/kg, wet weight) showed that this raptor died due to Pb poisoning. Pb concentration in the kidney was also high (Table S2). Furthermore, hair of sika deer was found in the stomach of the eagle, indicating this bird ate sika deer.

The ratio of Pb poisoning in adults and sub-adults was significantly higher than in juveniles of the Steller's sea eagle (Table 2). The white-tailed sea eagle showed similar

pattern of Pb accumulation depending on their ages, although not statistically significant. There were no significant differences between males and females ($p = 0.42$) in either the Steller's sea eagle or the white-tailed sea eagle (data not shown).

3.2. Pb concentrations in the liver, kidney, and blood samples s of raptors from Honshu and Shikoku

The liver sample of one golden eagle exceeded the level of Pb poisoning (Table 3). Hepatic Pb concentration of another golden eagle, one black kite, and one northern goshawk, and the blood Pb concentration of one golden eagle (0.14 mg/kg, wet weight) showed accumulation of high Pb concentrations, indicating Pb exposure. Although the number of kidney samples was limited, Pb level was almost the same between liver and kidney. Data regarding age, sex, and Pb concentrations (liver, kidney, and blood) are shown in Table S2.

3.3. Pb isotope ratios

The distributions of Pb isotope ratios ($^{208}Pb/^{206}Pb$, $^{207}Pb/^{206}Pb$) in various types of rifle bullets (1.90 – 2.10, 0.75 – 0.88), shot pellets (2.07 – 2.14, 0.85 – 0.87), and sinkers (2.08 – 2.20, 0.84 – 0.90), which were obtained from the shops in Japan, were reported in 2002. These materials had been purchased or collected from the carcasses or birds until 2001. It was confirmed that Pb isotope ratios of Pb rifle bullets, shot pellets and fishing sinkers used in 2015 by hunters or fishers in Japan were comparable (Fig. 2). Although it is difficult to distinguish between Pb shot pellets, slugs, hollow-point, air gun bullets, and sinkers due to the close distribution of Pb isotope ratios between them, Pb ratios from rifle bullets were almost distinct among ammunition. Pb isotope ratios of slugs

were different among three specimens, suggesting that the original source regions for the Pb present in these slugs were different.

We determined the Pb isotope ratios in the liver and the rifle bullets/shot pellets found in the stomach of the same individual (n = 4). In three sea eagles, Pb isotope ratios in the liver and the ammunition inside the stomach were comparable (Fig. 3), whereas one eagle showed different Pb isotope ratios between the liver and the ammunition (indicated in green, triangle). Fig. 4 shows the Pb isotope ratios in the liver of poisoned raptors.

The result shows that Steller's sea eagles were mainly poisoned by Pb rifle bullets, white-tailed sea eagles were poisoned by various types of ammunition or sinkers. Golden eagles were poisoned by Pb rifle bullets and other ammunition or sinkers, and black kite and northern goshawk were poisoned by Pb shot pellets, slugs, small rifle bullets, or sinkers (all data including normal Pb levels are shown in Fig. S2).

4. Discussion

In the present study, one quarter of the sea eagles from Hokkaido showed high Pb concentration of > 2 mg/kg wet weight in the liver, suggesting that these birds had been exposed to abnormally high Pb levels and suffered from Pb poisoning (Table 1). In addition, the Steller's sea eagle death in 2013 was suspected to be from Pb poisoning because X-ray and post-mortem examination showed that the stomach contained a rifle bullet fragment (Fig. 1). This eagle accumulated high Pb levels in both the liver and the kidney, indicating that these tissues would have had severe damage due to Pb exposure. Although more than 10 years have passed since the legislation regarding Pb ammunition was introduced, some hunters are still using Pb containing ammunition because they believe that Pb ammunition has stronger power than other types of ammunition, or the price of Pb ammunition is slightly inexpensive.

Pb poisoning in adults and sub-adults was higher than in juveniles of sea eagles (Table 2) because adults begin to consume their prey prior to juveniles. It means that adults have more opportunities to ingest Pb fragments, as they eat at locations on the carcasses of animals killed by Pb containing ammunition. Furthermore, Steller's sea eagles had a higher ratio of Pb poisoning than white-tailed sea eagles. This result may be due to several factors. First, although the sea eagles naturally consume fish, they have changed their major food source to deer carcasses in Hokkaido (Saito, 2009), and this tendency is particularly strong in the Steller's sea eagle. Second, as the body size of Steller's sea eagle is larger than that of the white-tailed sea eagle, it probably out-competes the white-tailed sea eagle at carcasses. This trend might be a reason for a higher risk of ingesting Pb ammunition in Steller's sea eagle compared to the other species. Naturally, sea eagles consume fish, they have opportunities to eat sika deer (*Cervus*

nippon yesoensis) carcasses in Hokkaido, because hunters leave carcasses or the deer are killed by trains (Saito, 2009). It is prohibited to leave the carcasses on the ground. However, some hunters take only a small portion of muscle to eat and leave the rest. Others take only one carcass and leave other carcasses due to limited human labor or a space in a light truck, although most hunters follow the regulation. Several cities give grants to hunters for culling harmful beasts. Therefore, hunters try to hunt sika deer as many as possible. There are also several hunters who think that it could be enough if they reduce the number of harmful beasts to protect farm products, and trees, and they leave the carcasses.

In 2014, the regulation was enforced in Hokkaido that prohibited the possession of Pb rifle bullets, slugs, or large shot pellets for hunting. Prior to this regulation, it was not illegal for hunters to keep Pb ammunition, but they were punished if they were found to use such ammunition. The new regulation aimed to improve this situation. However, the livers of four sea eagles showed high Pb concentrations at fatal levels and the blood of one mountain hawk eagle accumulated high concentration of Pb in 2015 (Table S2), indicating that the regulations are not yet effective or another path of Pb ingestion is possible.

The results from Honshu and Shikoku showed that Pb exposure in raptors also occurred in these areas (Table 3). The golden eagle, black kite, and most northern goshawks are resident birds. The results suggest that there is wider raptor Pb exposure in Japan. Many hunters use Pb ammunition for hunting wild animals, such as wild boar (*Sus scrofa*) or waterfowl. In Honshu, the Japanese black bear (*Ursus thibetanus japonicas*) was reported to accumulate high Pb concentrations, which suggests that they ingested Pb bullet or shot pellet fragments from their prey (Sato et al., 2007).

In Honshu and Shikoku, the use of Pb ammunition was restricted in certain locations, such as a wetland designated by the Ramsar Convention and its surrounding area. Moreover, Japan has conducted only a few studies of Pb poisoning of birds in these islands. Our results may only represent a fraction of the actual number of cases of Pb poisoning. The prevalence of Pb poisoning is not well known due to the shortage of data. Therefore, it is crucial to conduct further analyses of Pb concentrations in raptors and to determine the present state of Pb pollution, both in Hokkaido and in other parts of Japan.

Pb isotope ratios in the liver and the ammunition found in the stomach of the same individual showed that isotope ratios in liver would reflect those of ingested ammunition itself. One eagle had different Pb isotope ratios between the liver and ammunition (Fig. 3, indicated in green and triangle), indicated that this eagle had been exposed to Pb from one source (e.g. rifle bullets) and then subsequently ingested Pb from another source (e.g. shot pellets). Although Pb isotope ratios in the liver do not always indicate recently ingested ammunition, those in liver can be used to indicate the source of Pb exposure in many cases.

From the results of Pb isotope ratios in the liver of poisoned raptors, sea eagles were still contaminated by illegal Pb ammunition in Hokkaido (Fig. 4). The black kite also eats large animals, so it is also possible they were poisoned by rifle bullets. Therefore, all Pb ammunition and sinkers have a risk of causing Pb poisoning in all parts of Japan.

Some areas, such as California, have legislations against using Pb shot pellets as a means of combating Pb poisoning. Nevertheless, Pb poisoning persisted in the population of California condors (*Gymnogyps californianus*) despite a ban on Pb ammunition introduced in 2008 in some regions where condors had been reintroduced (Finkelstein et al., 2012). Therefore, an expanded legislation that requires hunters to use

non-lead ammunition was signed into law by the governor of California on October 11, 2013, for implementation no later than July 2019. As an alternative, Pb-free ammunition, such as Cu bullets, have almost the same efficiency for hunting as Pb ammunition (Knott et al., 2009; Thomas, 2013; Trinogga et al., 2013) and is less toxic to raptors (Franson et al., 2012). The modern international trend in hunting to use monolithic Cu or brass rifle bullets for large animals will likely take a long time to replace Pb rifle bullets due to higher cost.

5. Conclusions

In the present study, one quarter of the sea eagles from Hokkaido showed high Pb concentrations, suggesting that these birds were exposed to abnormally high Pb levels and suffered from Pb poisoning although more than 10 years have passed since the regulation was introduced. In other areas of Japan, both surveillance and regulation were less strict than in Hokkaido, and there was also Pb exposure in raptors in these areas. In addition, Pb isotope ratios showed that about half of Pb-poisoned raptors, mainly endangered Steller's sea eagles and white-tailed sea eagles, were exposed to the possible illegal use of Pb rifle bullets in Hokkaido. The number of identified cases of Pb poisoning in raptors found dead could be only a fraction of the actual cases because of the wildlife behavior. Therefore, it is necessary to accurately determine the situation to develop appropriate regulations for the conservation of wild birds.

Table 1. Hepatic Pb levels (mg/kg, wet weight, range) and the assesments of Pb exposure in raptors from Hokkaido after the regulation.

Species	Sample size	Pb concentration in liver	Assessments		
		mg/kg, wet wt, median, (range)	Pb poisoning (> 2.0 mg.kg)	High Pb exposure (0.2 - 2.0 mg/kg)	Non toxic (< 0.2 mg/kg)
Steller's sea eagle	43	0.12 (ND - 36.6)	18	2	23
White-tailed sea eagle	50	0.06 (ND - 56.4)	12	4	34
Blakiston's fish owl	13	0.01 (ND - 0.04)	0	0	13
Mountain hawk eagle	7	0.01 (ND - 0.09)	0	0	7
Northern goshawk	6	0.01 (ND - 0.04)	0	0	6
Sparrowhawk	2	- (ND - 0.04)	0	0	2
Peregrine falcon, Black kite	1, each species	0.07 (0.01 - 0.12)	0	0	2

ND: non detectable (The limit of detection for Pb was 0.01 μg/L in digested solution.)

Table 2. Hepatic concentrations of Pb depending on age in Steller's sea eagle and White-tailed sea eagle.

Species	Age	Sample size	Pb concentration in liver mg/kg, wet wt, median, (range)
Steller's sea eagle	ad.	22	2.5 (ND – 36.6) †
	sub.	8	17.2 (0.02 – 23.8) ‡
	juv.	13	0.01 (ND – 3.7)†, ‡
	unk.	0	
White-tailed sea eagle	ad.	18	0.1 (0.01 – 53.7)
	sub.	19	0.1 (ND – 56.4)
	juv.	10	0.02 (ND – 10.8)
	unk.	3	0.1 (ND – 0.24)

ad.: adult; sub.: sub-adult; juv.: juvenile; unk.: unknown; ND: non detectable (The limit of detection for Pb was 0.01 μg/L in digested solution.)

Steel-Dwass test for each age was used.

†: Significantly ($p < 0.01$) different between adults and juvenile in Steller's sea eagle.

‡: Significantly ($p < 0.01$) different between sub-adults and juvenile in Steller's sea eagle.

Table 3. Hepatic Pb levels (mg/kg, wet weight, range) and the assesments of Pb exposure in raptors from Honshu and Shikoku.

Species	Sample size	Pb concentration in liver	Assessments		
		mg/kg, wet wt, median, (range)	Pb poisoning (> 2.0 mg.kg)	High Pb exposure (0.2 - 2.0 mg/kg)	Non toxic (< 0.2 mg/kg)
Golden eagle	13	0.04 (0.01 - 2.3)	1	1	11
Northern goshawk	9	0.02 (0.01 - 0.35)	0	1	8
Black kite	9	0.05 (0.02 - 0.42)	0	1	8
Ural owl	4	0.02 (0.01 - 0.03)	0	0	4
Sparrowhawk	2	0.04 (0.02 - 0.06)	0	0	2
Brown hawk owl	2	0.08 (0.02 - 0.13)	0	0	2
Mountain hawk eagle, Peregrine falcon, Osprey, Grey-faced buzzard, Japanese sparrowhawk, Common kestrel, Sunda scops owl	1, each species	0.01 (ND - 0.04)	0	0	7

ND: non detectable (The limit of detection for Pb was 0.01 μg/L in digested solution.)

Fig. 1. A X-ray photograph (ventrodorsal) of the Steller's sea eagle specimen that died in 2013 (provided by the Institute for Raptor Biomedicine Japan (IRBJ)). The stomach contained a bullet fragment as indicated with the white arrow.

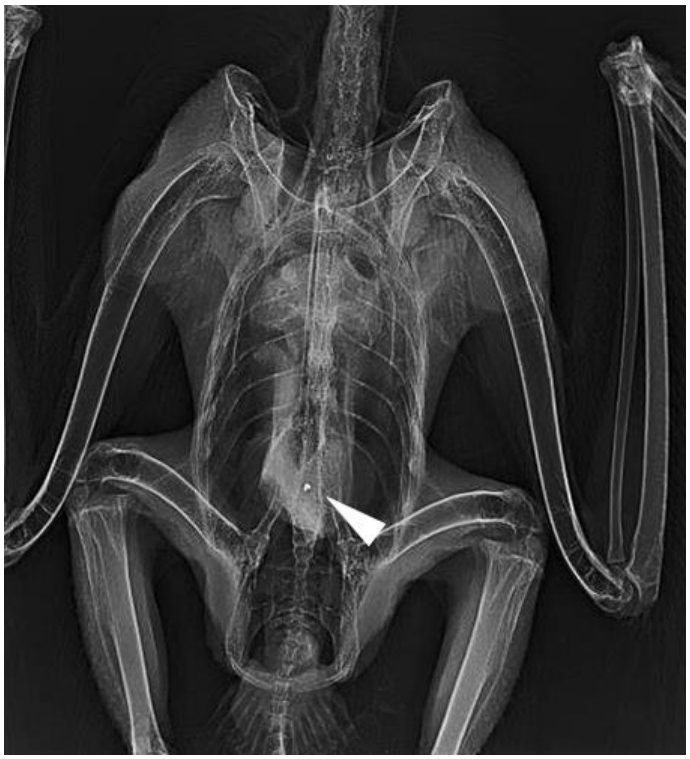


Fig. 2. Pb isotope ratios in ammunition currently being used.

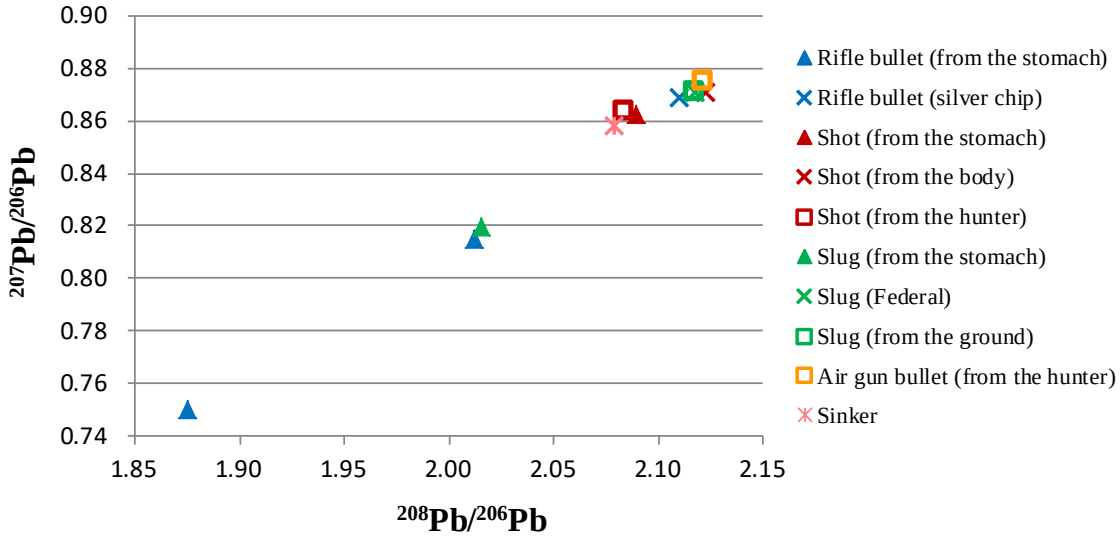
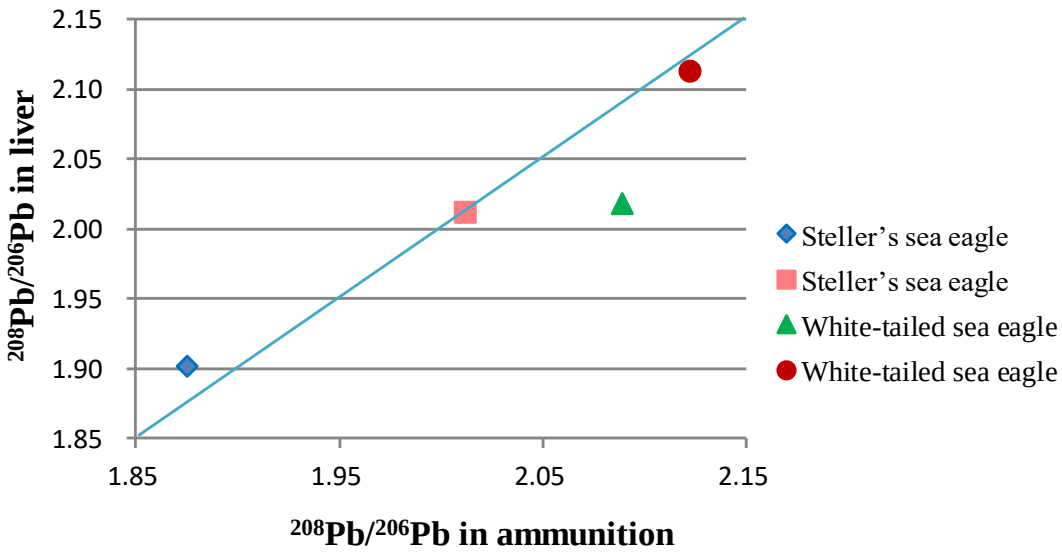


Fig. 3. Comparison of Pb isotope ratios of $^{208}Pb/^{206}Pb$ (a) or $^{207}Pb/^{206}Pb$ (b) between the liver and the ammunition found in the stomach from the same individual of raptors. Blue lines show the same ratios of $^{208}Pb/^{206}Pb$ between liver and ammunition

(a)



(b)

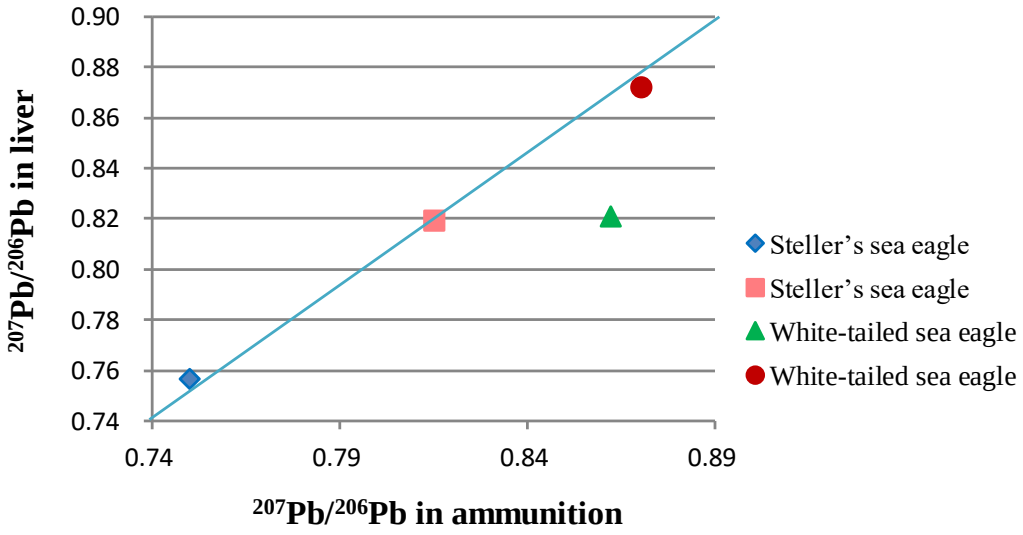
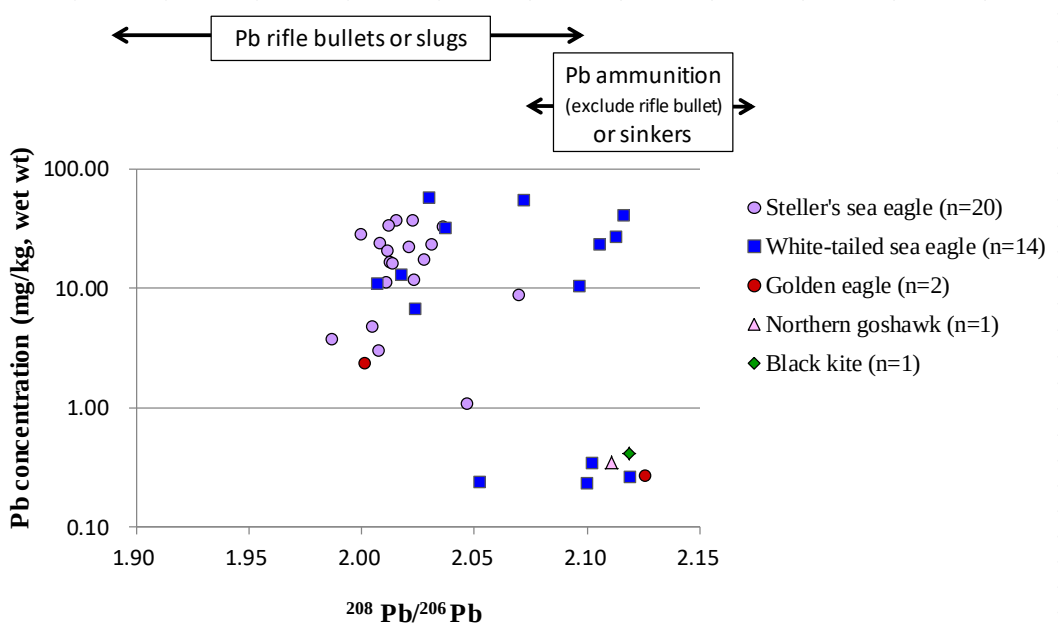


Fig. 4. Comparison of Pb levels and Pb isotope ratio ($^{208}Pb/^{206}Pb$, RSD < 0.5) in the liver of raptors that had high Pb concentration (> 0.2 mg/kg, wet weight). Comparison of Pb levels and $^{208}Pb/^{206}Pb$ or $^{207}Pb/^{206}Pb$ of all samples including normal Pb levels are shown in Fig. S2.



References

Berny, P., Vilagines, L., Cugnasse, J.-M., Mastain, O., Chollet, J.-Y., Joncour, G., Razin, M., 2015. VIGILANCE POISON: Illegal poisoning and lead intoxication are the main factors affecting avian scavenger survival in the Pyrenees (France). Ecotoxicol. Environ. Saf. 118, 71–82.

Church, M.E., Gwiazda, R., Risebrough, R.W., Sorenson, K., Chamberlain, C.P., Farry, S., Heinrich, W., Rideout, B.A., Smith, D.R., 2006. Ammunition is the principal source of lead accumulated by California condors re-introduced to the wild. Environ. Sci. Technol. 40, 6143–6150.

Finkelstein, M.E., Doak, D.F., George, D., Burnett, J., Brandt, J., Church, M., Grantham, J., Smith, D.R., 2012. Lead poisoning and the deceptive recovery of the critically endangered California condor. Proc. Natl. Acad. Sci. 109, 11449-11454. doi:10.1073/pnas.1203141109

Finkelstein, M.E., Kuspa, Z.E., Welch, A., Eng, C., Clark, M., Burnett, J., Smith, D.R., 2014. Linking cases of illegal shootings of the endangered California condor using stable lead isotope analysis. Environ. Res. 134, 270–279.

Fisher, I.J., Pain, D.J., Thomas, V.G., 2006. A review of lead poisoning from ammunition sources in terrestrial birds. Biol. Conserv. 131, 421–432. doi:http://dx.doi.org/10.1016/j.biocon.2006.02.018

Franson, J.C., Lahner, L.L., Meteyer, C.U., Rattner, B.A., 2012. Copper pellets simulating oral exposure to copper ammunition: absence of toxicity in American kestrels (*Falco sparverius*). Arch. Environ. Contam. Toxicol. 62, 145–153.

Haig, S.M., D'Elia, J., Eagles-Smith, C., Fair, J.M., Gervais, J., Herring, G., Rivers, J.W., Schulz, J.H., 2014. The persistent problem of lead poisoning in birds from ammunition and fishing tackle. Condor 116, 408–428. doi:10.1650/CONDOR-14-36.1

Iwata, H., Watanabe, M., Kim, E.-Y., Gotoh, R., Yasunaga, G., Tanabe, S., Masuda, Y., Fujita, S., 2000. Contamination by chlorinated hydrocarbons and lead in Steller's Sea Eagle and White-tailed Sea Eagle from Hokkaido, Japan, in: First Symposium on Stellar's and White-Tailed Sea Eagles in East Asia. Wild Bird Society of Japan, Tokyo. pp. 91–106.

Kendall, R.J., Lacker, T.E., Bunck, C., Daniel, B., Driver, C., Grue, C.E., Leighton, F., Stansley, W., Watanabe, P.G., Whitworth, M., 1996. An ecological risk assessment of lead shot exposure in non-waterfowl avian species: Upland game birds and raptors. Environ. Toxicol. Chem. 15, 4–20. doi:10.1002/etc.5620150103

Kim, E.-Y., Goto, R., Iwata, H., Masuda, Y., Tanabe, S., Fujita, S., 1999. Preliminary survey of lead poisoning of Steller's sea eagle (*Haliaeetus pelagicus*) and white-tailed sea eagle (*Haliaeetus albicilla*) in Hokkaido, Japan. Environ. Toxicol. Chem. 18, 448–451. doi:10.1002/etc.5620180312

Knott, J., Gilbert, J., Green, R.E., Hoccom, D.G., 2009. Comparison of the lethality of lead and copper bullets in deer control operations to reduce incidental lead poisoning; field trials in England and Scotland. Conserv. Evid. 6, 71–78.

Komárek, M., Ettler, V., Chrastný, V., Mihaljevič, M., 2008. Lead isotopes in environmental sciences: a review. Environ. Int. 34, 562–577.

Kurosawa, N., 2000. Lead poisoning in Steller's sea eagles and white-tailed sea eagles, in: First Symposium on Stellar's and White-Tailed Sea Eagles in East Asia. Wild Bird Society of Japan, Tokyo. pp. 107–109.

Kyodo, 2015. The Japan Times NEWS Website; http://www.japantimes.co.jp/news/2015/03/16/national/japanese-golden-eagles-face-extinction-as-numbers-dive [WWW Document]. Japan Times NEWS Website. URL http://www.japantimes.co.jp/news/2015/03/16/national/japanese-golden-eagles-face-extinction-as-numbers-dive/

Langner, H.W., Domenech, R., Slabe, V.A., Sullivan, S.P., 2015. Lead and Mercury in Fall Migrant Golden Eagles from Western North America. Arch. Environ. Contam. Toxicol. 1–8.

Madry, M.M., Kraemer, T., Kupper, J., Naegeli, H., Jenny, H., Jenni, L., Jenny, D., 2015. Excessive lead burden among golden eagles in the Swiss Alps. Environ. Res. Lett. 10, 034003.

Martinez-Haro, M., Taggart, M.A., Martín-Doimeadiós, R.R.C., Green, A.J., Mateo, R., 2011. Identifying Sources of Pb Exposure in Waterbirds and Effects on Porphyrin Metabolism Using Noninvasive Fecal Sampling. Environ. Sci. Technol. 45, 6153–6159. doi:10.1021/es2009242

Mateo, R., 2009. Lead poisoning in wild birds in Europe and the regulations adopted by different countries. Ingestion lead from spent Ammunit. Implic. Wildl. humans 2009, 71–98.

Nakata, H., Nakayama, S.M.M., Ikenaka, Y., Mizukawa, H., Ishii, C., Yohannes, Y.B.,

Konnai, S., Darwish, W.S., Ishizuka, M., 2015. Metal extent in blood of livestock from Dandora dumping site, Kenya: Source identification of Pb exposure by stable isotope analysis. Environ. Pollut. 205, 8–15.

Pain, D.J., Amiardtriquet, C., 1993. Lead Poisoning of Raptors in France and Elsewhere. Ecotoxicol. Environ. Saf. 25, 183–192. doi:http://dx.doi.org/10.1006/eesa.1993.1017

Pain, D.J., Carter, I., Sainsbury, A.W., Shore, R.F., Eden, P., Taggart, M.A., Konstantinos, S., Walker, L.A., Meharg, A.A., Raab, A., 2007. Lead contamination and associated disease in captive and reintroduced red kites (*Milvus milvus*) in England. Sci. Total Environ. 376, 116–127.

Pain, D.J., Sears, J., Newton, I., 1995. Lead concentrations in birds of prey in Britain. Environ. Pollut. 87, 173–180. doi:http://dx.doi.org/10.1016/0269-7491(94)P2604-8

Rattner, B.A., 2009. History of wildlife toxicology. Ecotoxicology 18, 773–783.

Saito, K., 2009. Lead poisoning of Steller's Sea-Eagle (*Haliaeetus pelagicus*) and Whitetailed Eagle (*Haliaeetus albicilla*) caused by the ingestion of lead bullets and slugs. Hokkaido Japan. RT Watson, M. Fuller, M. Pokras, WG Hunt (Eds.). Ingestion Lead from Spent Ammunit. Implic. Wildl. Humans. Peregrine Fund, Boise, Idaho, USA.

Sato, I., Tsujimoto, T., Yamashita, T., Saita, E., Watanabe, G., Taya, K., Sera, K., Tsuda, S., 2007. A Survey on Contamination with Cadmium, Thallium and Lead in Wild Fauna. J. Japan Vet. Med. Assoc. 60, 733–737. doi:10.12935/jvma1951.60.733

Scheuhammer, A.M., Templeton, D.M., 1998. Use of stable isotope ratios to distinguish sources of lead exposure in wild birds. Ecotoxicology 7, 37–42.

Thomas, V.G., 2013. Lead-Free Hunting Rifle Ammunition: Product Availability, Price, Effectiveness, and Role in Global Wildlife Conservation. Ambio 42, 737–745. doi:10.1007/s13280-012-0361-7

Trinogga, A., Fritsch, G., Hofer, H., Krone, O., 2013. Are lead-free hunting rifle bullets as effective at killing wildlife as conventional lead bullets? A comparison based on wound size and morphology. Sci. Total Environ. 443, 226–232.

Vallverdú-Coll, N., Mougeot, F., Ortiz-Santaliestra, M.E., Castaño, C., Santiago-Moreno, J., Mateo, R., 2016. Effects of Lead Exposure on Sperm Quality and Reproductive Success in an Avian Model. Environ. Sci. Technol. 50, 12484–12492.

Yabe, J., Nakayama, S.M.M., Ikenaka, Y., Yohannes, Y.B., Bortey-Sam, N., Oroszlany, B., Muzandu, K., Choongo, K., Kabalo, A.N., Ntapisha, J., Mweene, A., Umemura, T., Ishizuka, M., 2015. Lead poisoning in children from townships in the vicinity of a lead-zinc mine in Kabwe, Zambia. Chemosphere 119, 941–947. doi:10.1016/j.chemosphere.2014.09.028

Supplementary data

Table S1. Detailed analytical conditions of ICP-MS (7700 series, Agilent technologies) for analysis of Pb concentration (a) and Pb isotope ratios (b).

Table S2. Sample information and Pb concentration (dry and wet weight).

Table S3. Comparison of Pb concentrations in the liver (mg/kg dry weight) of eagles in Hokkaido with previous data.

Fig. S1. Map of sampling areas in Japan.

Fig. S2. Comparison of Pb levels and Pb isotope ratio of $^{208}Pb/^{206}Pb$ (a) or $^{207}Pb/^{206}Pb$ (b) of all samples including normal Pb levels that RSD values were less than 0.5.

Table S1. Detailed analytical conditions of ICP-MS (7700 series, Agilent technologies) for analysis of Pb concentration (a) and Pb isotope ratios (b).

Instrument types are as follows. Regression type; Linear equation, Auto sampler type; Agilent I-AS, Nebulizer type; Micro Mist, and Spray chamber type; Scott.

(a)

Parameter	Value
RF Power	1500 W
Argon gas pressure	600 kPa
Cell gas (Helium)	100 kPa
Peak pattern	1
Replicates	3
Sweeps/replicate	100
Stabilization time	30 s

(b)

Parameter	Value
RF Power	1500 W
Argon gas pressure	600 kPa
Cell gas (Helium)	100 kPa
Peak pattern	3
Replicates	10
Sweeps/replicate	1000
Integration time/mass	9.00 s
Stabilization time	40 s

Table S2. Sample information and Pb concentration (dry and wet weight).

m: male; f: female; ad.: adult; sub.: sub-adult; juv.: juvenile; ch.: chick; emb.: embryo; unk.: unknown; ND: non detectable (The limit of detection for Pb was 0.01 μg/L in digested solution.)

a) Liver and kidney samples collected before the regulation in Hokkaido.

b) Liver and kidney samples collected after the regulation in Hokkaido.

c) Liver and kidney samples collected in Honshu and Shikoku.

d) Blood samples collected in Hokkaido and Honshu

a)

Year	Species	Sex	Age	Liver (mg/kg) wet wt	Kidney (mg/kg) wet wt	Assessments
1998	Steller's sea eagle	unk.	unk.	0.29	0.01	high Pb exposure
1999	Steller's sea eagle	m	ad.	0.03	0.01	non-toxic
1999	Steller's sea eagle	f	sub.	27.9	9.88	Pb poisoning
1999	Steller's sea eagle	f	ad.	11.0	4.04	Pb poisoning
1999	White-tailed sea eagle	f	ad.	0.02	0.06	non-toxic

b)

Year	Species	Sex	Age	Liver (mg/kg) wet wt.	Kidney (mg/kg) wet wt.	Assessments
2008	Steller's sea eagle	m	ad.	16.55	4.93	Pb poisoning
2008	Steller's sea eagle	m	sub.	8.65	3.31	Pb poisoning
2009	Steller's sea eagle	m	juv.	0.03	0.03	non toxic
2010	Steller's sea eagle	unk.	ad.	4.68	0.68	Pb poisoning
2010	Steller's sea eagle	m	sub.	23.81	4.19	Pb poisoning
2010	Steller's sea eagle	m	sub.	22.02	—	Pb poisoning

2011	Steller's sea eagle	m	ad.	0.22	0.16	high Pb exposure
2011	Steller's sea eagle	unk.	ad.	0.05	—	non toxic
2011	Steller's sea eagle	unk.	ad.	36.56	8.2	Pb poisoning
2011	Steller's sea eagle	f	ad.	32.36	11.02	Pb poisoning
2012	Steller's sea eagle	f	juv.	0.01	0.01	non toxic
2012	Steller's sea eagle	unk.	juv.	0.04	0.05	non toxic
2013	Steller's sea eagle	f	juv.	0.02	0.02	non toxic
2013	Steller's sea eagle	f	juv.	0.01	—	non toxic
2013	Steller's sea eagle	f	ad.	0.01	0.03	non toxic
2013	Steller's sea eagle	f	juv.	0.01	0.15	non toxic
2013	Steller's sea eagle	f	sub.	17.16	3	Pb poisoning
2013	Steller's sea eagle	m	ad.	0.05	0.07	non toxic
2013	Steller's sea eagle	m	sub.	0.04	0.03	non toxic
2013	Steller's sea eagle	f	ad.	15.94	5.34	Pb poisoning
2013	Steller's sea eagle	f	ad.	22.89	—	Pb poisoning
2013	Steller's sea eagle	unk.	juv.	2.99	4.91	Pb poisoning
2013	Steller's sea eagle	unk.	juv.	3.71	—	Pb poisoning
2013	Steller's sea eagle	m	ad.	0.06	0.1	non toxic
2013	Steller's sea eagle	m	ad.	36.29	14.72	Pb poisoning
2014	Steller's sea eagle	m	ad.	0.03	0.02	non toxic
2014	Steller's sea eagle	m	ad.	0	0	non toxic
2014	Steller's sea eagle	unk.	juv.	0	—	non toxic
2014	Steller's sea eagle	f	juv.	0.01	0	non toxic
2014	Steller's sea eagle	unk.	juv.	0	0	non toxic
2014	Steller's sea eagle	unk.	ad.	0.02	0.02	non toxic
2014	Steller's sea eagle	unk.	ad.	11.55	—	Pb poisoning
2014	Steller's sea eagle	m	ad.	0.17	0.08	non toxic
2014	Steller's sea eagle	unk.	juv.	1.08	0.19	high Pb exposure
2014	Steller's sea eagle	unk.	ad.	15.32	2.1	Pb poisoning
2014	Steller's sea eagle	f	ad.	0.01	0	non toxic
2014	Steller's sea eagle	unk.	sub.	0.02	0.01	non toxic

2014	Steller's sea eagle	f	ad.	18.33	4.65	Pb poisoning
2014	Steller's sea eagle	unk.	juv.	N.D.	0	non toxic
2014	Steller's sea eagle	f	ad.	0.01	0.01	non toxic
2015	Steller's sea eagle	m	sub.	20.46	8.01	Pb poisoning
2015	Steller's sea eagle	f	ad.	32.94	8.47	Pb poisoning
2015	Steller's sea eagle	m	juv.	0.01	0.01	non toxic
2007	White-tailed sea eagle	f	sub.	0.11	0.34	non toxic
2008	White-tailed sea eagle	f	ad.	23.3	14	Pb poisoning
2009	White-tailed sea eagle	m	ad.	53.74	8.48	Pb poisoning
2010	White-tailed sea eagle	f	sub.	22.05	1.32	Pb poisoning
2011	White-tailed sea eagle	unk.	ad.	0.06	0.08	non toxic
2011	White-tailed sea eagle	f	juv.	10.83	9.12	Pb poisoning
2011	White-tailed sea eagle	m	sub.	0.02	0.02	non toxic
2011	White-tailed sea eagle	m	juv.	0.02	0.03	non toxic
2011	White-tailed sea eagle	m	sub.	0.02	0.02	non toxic
2011	White-tailed sea eagle	f	sub.	31.56	9.06	Pb poisoning
2011	White-tailed sea eagle	m	ad.	0.03	0.02	non toxic
2011	White-tailed sea eagle	m	unk.	0.05	0.04	non toxic
2011	White-tailed sea eagle	unk.	sub.	0.07	—	non toxic
2011	White-tailed sea eagle	unk.	ad.	0.02	—	non toxic
2011	White-tailed sea eagle	f	sub.	6.72	6.22	Pb poisoning
2012	White-tailed sea eagle	unk.	sub.	0.26	0.48	high Pb exposure
2012	White-tailed sea eagle	f	sub.	0.03	0.1	non toxic
2012	White-tailed sea eagle	unk.	sub.	0.03	0.05	non toxic
2012	White-tailed sea eagle	f	ad.	0.34	1.14	high Pb exposure
2013	White-tailed sea eagle	unk.	juv.	10.25	6.87	Pb poisoning
2013	White-tailed sea eagle	unk.	juv.	0	0.02	non toxic
2013	White-tailed sea eagle	unk.	ad.	0.02	0.01	non toxic
2013	White-tailed sea eagle	m	sub.	56.41	8.39	Pb poisoning
2013	White-tailed sea eagle	unk.	juv.	0.01	0.02	non toxic
2013	White-tailed sea eagle	unk.	sub.	0	—	non toxic

2013	White-tailed sea eagle	m	ad.	0.03	0.03	non toxic
2013	White-tailed sea eagle	unk.	juv.	0	0.02	non toxic
2013	White-tailed sea eagle	unk.	juv.	0.17	0.15	non toxic
2014	White-tailed sea eagle	unk	ad.	0.06	0.05	non toxic
2014	White-tailed sea eagle	m	sub.	0.02	0.03	non toxic
2014	White-tailed sea eagle	unk.	unk.	N.D.	N.D.	non toxic
2014	White-tailed sea eagle	unk.	unk.	0.24	0.07	high Pb exposure
2014	White-tailed sea eagle	f	sub.	0.23	0.07	high Pb exposure
2014	White-tailed sea eagle	f	sub.	0.09	0.06	non toxic
2014	White-tailed sea eagle	m	ad.	0.01	0.01	non toxic
2014	White-tailed sea eagle	unk.	ad.	12.85	7.19	Pb poisoning
2014	White-tailed sea eagle	m	sub.	23.44	1.46	Pb poisoning
2014	White-tailed sea eagle	m	juv.	0.04	0.07	non toxic
2014	White-tailed sea eagle	unk.	juv.	0.01	0.01	non toxic
2014	White-tailed sea eagle	m	ad.	0.09	0.07	non toxic
2014	White-tailed sea eagle	unk.	ad.	0.12	0.13	non toxic
2014	White-tailed sea eagle	m	sub.	0.03	0.06	non toxic
2015	White-tailed sea eagle	m	ad.	40.23	6.74	Pb poisoning
2015	White-tailed sea eagle	f	ad.	26.82	4.81	Pb poisoning
2015	White-tailed sea eagle	f	ad.	0.02	0.02	non toxic
2015	White-tailed sea eagle	m	ad.	0.07	0.08	non toxic
2015	White-tailed sea eagle	f	sub.	0.06	0.05	non toxic
2015	White-tailed sea eagle	f	juv.	0.01	0.01	non toxic
2015	White-tailed sea eagle	f	sub.	0.01	0.01	non toxic
2015	White-tailed sea eagle	f	ad.	0.02	0.03	non toxic
unk.	Blakiston's fish owl	f	ad.	0.01	0.01	non toxic
unk.	Blakiston's fish owl	m	juv.	N.D.	0.01	non toxic
unk.	Blakiston's fish owl	m	ad.	N.D.	N.D.	non toxic
unk.	Blakiston's fish owl	f	juv.	N.D.	0.01	non toxic
unk.	Blakiston's fish owl	f	juv.	0	0.01	non toxic
unk.	Blakiston's fish owl	f	ch.	N.D.	N.D.	non toxic

unk.	Blakiston's fish owl	m	sub.	N.D.	N.D.	non toxic
unk.	Blakiston's fish owl	m	ad.	0.02	0.02	non toxic
unk.	Blakiston's fish owl	m	juv.	0.02	0.03	non toxic
unk.	Blakiston's fish owl	m	ad.	0.02	0.01	non toxic
unk.	Blakiston's fish owl	m	sub.	0.01	0.01	non toxic
unk.	Blakiston's fish owl	m	ad.	0.01	0.01	non toxic
2014	Blakiston's fish owl	f	juv.	0.04	0.02	non toxic
2011	Mountain hawk eagle	unk.	juv.	0.01	0	non toxic
2012	Mountain hawk eagle	unk.	juv.	N.D.	0	non toxic
2012	Mountain hawk eagle	m	sub.	0.01	0.02	non toxic
2012	Mountain hawk eagle	unk.	ad.	0.09	0.06	non toxic
2013	Mountain hawk eagle	unk.	sub.	N.D.	0	non toxic
2014	Mountain hawk eagle	unk.	ad.	0.04	0.01	non toxic
2014	Mountain hawk eagle	f	ad.	0.02	0.04	non toxic
unk.	Northern goshawk	unk.	juv.	0.03	0.01	non toxic
unk.	Northern goshawk	unk.	ad.	0	0	non toxic
unk.	Northern goshawk	unk.	sub.	0.02	0.03	non toxic
unk.	Northern goshawk	m	juv.	0.02	0.02	non toxic
unk.	Northern goshawk	f	ad.	0.01	0.01	non toxic
2014	Northern goshawk	unk.	juv.	0.01	0.01	non toxic
2012	Sparrowhawk	f	juv.	N.D.	0	non toxic
2015	Sparrowhawk	f	ad.	0	0	non toxic
unk.	Peregrine falcon	unk.	juv.	0.12	0.32	non toxic
2014	Black kite	unk.	sub.	0.01	0.01	non toxic

c)

year	species	sex	age	Liver (mg/kg) wet wt.	Kidney (mg/kg) wet wt.	Assessments
1993	Golden eagle	m	sub.	0.03	—	non toxic
1994	Golden eagle	unk.	unk.	0.03	—	non toxic

1995	Golden eagle	m	sub.	2.31	—	Pb poisoning
1998	Golden eagle	unk.	juv.	0.14	—	non toxic
1999	Golden eagle	unk.	ch.	0.03	—	non toxic
2000	Golden eagle	unk.	unk.	0.07	—	non toxic
2000	Golden eagle	unk.	unk.	0.02	—	non toxic
2002	Golden eagle	unk.	emb.	0.04	—	non toxic
2002	Golden eagle	f	emb.	0.01	—	non toxic
2003	Golden eagle	unk.	juv.	0.12	—	non toxic
2003	Golden eagle	f	unk.	0.03	—	non toxic
2004	Golden eagle	m	unk.	0.05	—	non toxic
2004	Golden eagle	m	ch.	0.27	—	high Pb exposure
1996	Northern goshawk	unk.	unk.	0.02	0.04	non toxic
1999	Northern goshawk	unk.	unk.	0.35	0.21	high Pb exposure
2008	Northern goshawk	f	ad.	0.02	—	non toxic
2009	Northern goshawk	f	ad.	0.02	—	non toxic
2009	Northern goshawk	m	juv.	0.02	—	non toxic
2009	Northern goshawk	f	ad.	0.01	—	non toxic
2010	Northern goshawk	f	juv.	0.06	—	non toxic
2011	Northern goshawk	unk.	unk.	0.01	—	non toxic
2012	Northern goshawk	unk.	unk.	0.03	—	non toxic
2007	Black kite	m	ad.	0.02	—	non toxic
2008	Black kite	f	ad.	0.03	—	non toxic
2008	Black kite	m	ad.	0.05	—	non toxic
2008	Black kite	f	ad.	0.42	—	high Pb exposure
2008	Black kite	f	ad.	0.02	—	non toxic
2010	Black kite	f	ad.	0.07	—	non toxic
2010	Black kite	m	ad.	0.12	—	non toxic
2012	Black kite	unk.	unk.	0.11	—	non toxic
2013	Black kite	unk.	unk.	0.04	—	non toxic
2012	Ural owl	unk.	unk.	0.02	—	non toxic

2012	Ural owl	unk.	unk.	0.01	—	non toxic
2012	Ural owl	unk.	unk.	0.01	—	non toxic
2012	Ural owl	unk.	unk.	0.03	—	non toxic
2010	Sparrowhawk	unk.	unk.	0.02	—	non toxic
2013	Sparrowhawk	unk.	unk.	0.06	—	non toxic
2013	Brown hawk owl	unk.	unk.	0.13	—	non toxic
2013	Brown hawk owl	unk.	unk.	0.02	—	non toxic
2006	Mountain hawk eagle	m	ad.	0.02	—	non toxic
2006	Peregrine falcon	unk.	unk.	0.04	0.03	non toxic
2011	Osprey	unk.	unk.	0.01	0.01	non toxic
2012	Grey-faced buzzard	unk.	unk.	0.05	—	non toxic
2012	Japanese sparrowhawk	unk.	unk.	0	—	non toxic
2013	Common kestrel	unk.	unk.	0.01	—	non toxic
2012	Sunda scops owl	unk.	unk.	0.01	—	non toxic

d)

Year	Species	Place	Sex	Age	Blood (mg/kg, wet wt)	Assessments
2011	White-tailed sea eagle	Hokkaido	unk.	unk.	0.16	high Pb exposure
2013	Golden eagle	Honshu	unk.	unk.	0.14	high Pb exposure
2015	Mountain hawk eagle	Hokkaido	unk.	unk.	0.38	high Pb exposure

Table S3. Comparison of Pb concentrations in the liver (mg/kg dry weight) of eagles in Hokkaido with previous data.

Reference: Iwata et al., 2000. “Contamination by chlorinated hydrocarbons and lead in Steller’s sea eagle and white-tailed sea eagle from Hokkaido, Japan.”

Species	Year	Pb concentration (mg/kg, dry wt)	Assessments	References
Steller’s sea eagle	1986	0.06	non-toxic	(Iwata et al., 2000)
Steller’s sea eagle	1995	75	Pb poisoning	(Iwata et al., 2000)
Steller’s sea eagle	1997	139	Pb poisoning	(Iwata et al., 2000)
White-tailed sea eagle	1997	79	Pb poisoning	(Iwata et al., 2000)
White-tailed sea eagle	1997	77	Pb poisoning	(Iwata et al., 2000)
Steller’s sea eagle	1998	232	Pb poisoning	(Iwata et al., 2000)
Steller’s sea eagle	1998	0.21	non-toxic	(Iwata et al., 2000)
Steller’s sea eagle	1998	0.11	non-toxic	(Iwata et al., 2000)
Steller’s sea eagle	1998	0.9	high Pb exposure	This study
White-tailed sea eagle	1998	174	Pb poisoning	(Iwata et al., 2000)
Steller’s sea eagle	1999	74.9	Pb poisoning	This study
Steller’s sea eagle	1999	32.0	Pb poisoning	This study
Steller’s sea eagle	1999	0.07	non-toxic	This study
White-tailed sea eagle	1999	0.05	non-toxic	This study

Fig. S1. Map of sampling areas in Japan.

In the case of Hokkaido, the places of six national parks were shown by green circles.

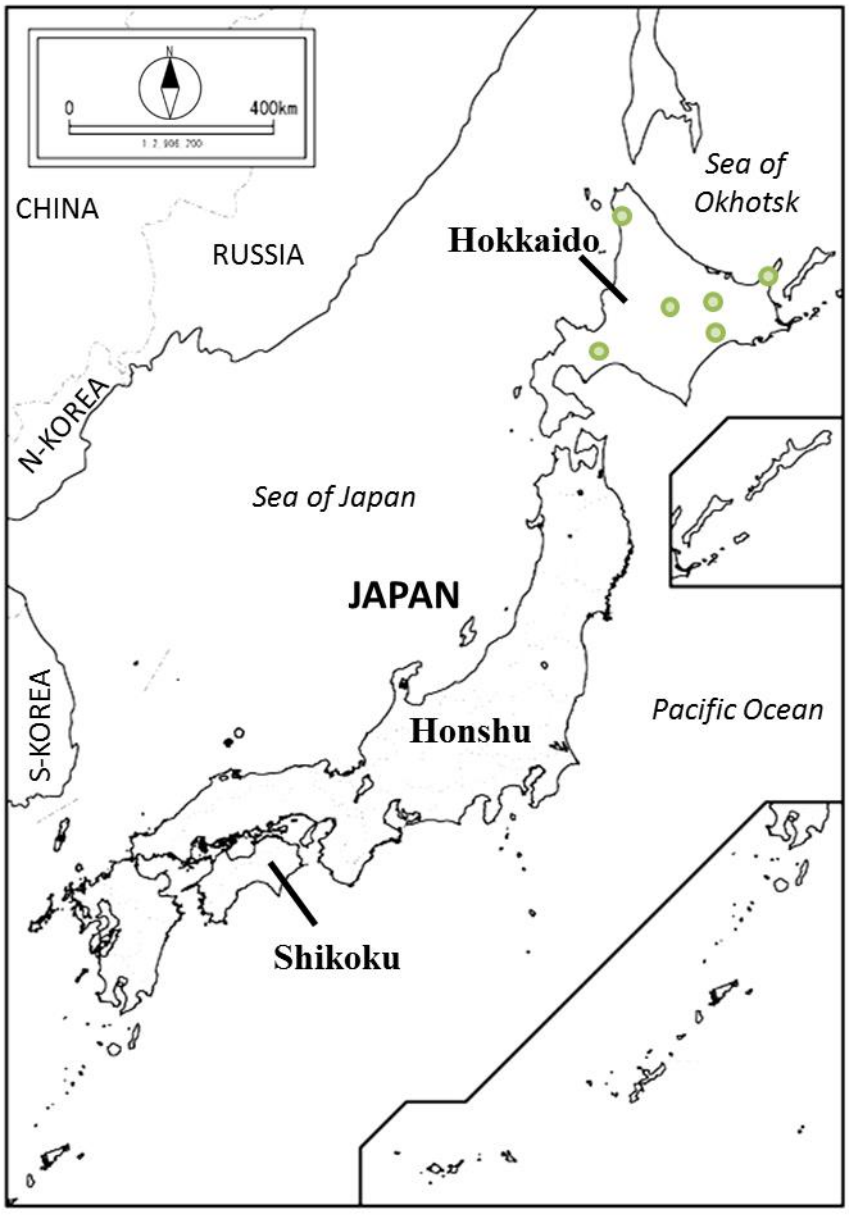
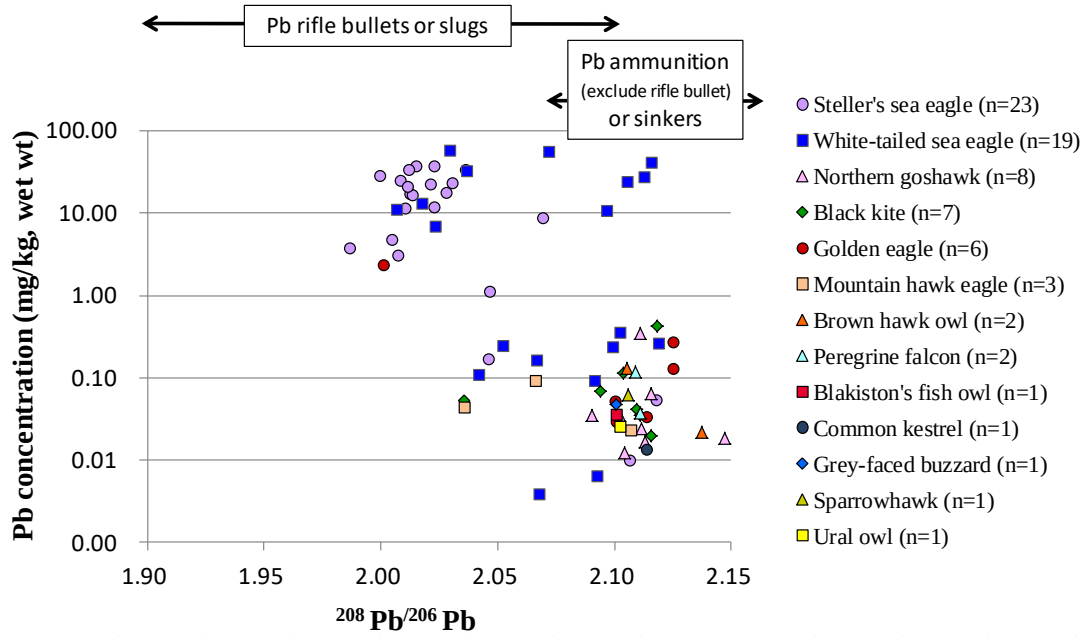
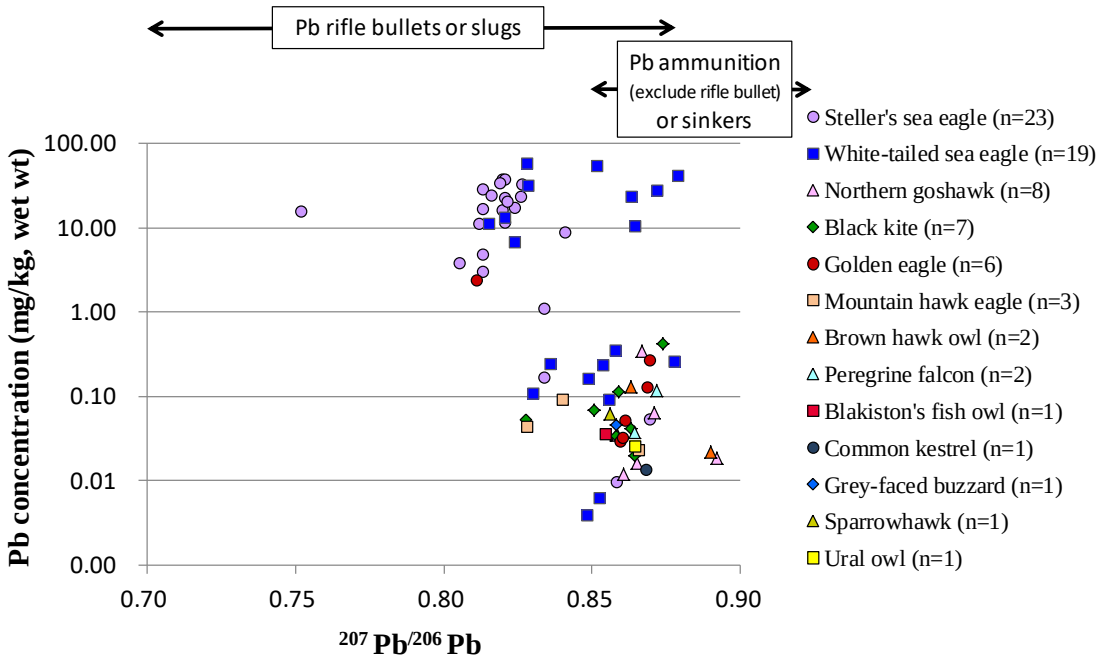


Fig. S2. Comparison of Pb levels and Pb isotope ratio of $^{208}Pb/^{206}Pb$ (a) or $^{207}Pb/^{206}Pb$ (b) of all samples including normal Pb levels that RSD values were less than 0.5.

(a)



(b)



Note 2-1

Pb concentration in liver and kidney by dry weight (linked to the chapter 2)

Background and Objective

As for the chapter 2, Pb concentrations by dry weight were not shown. However, Pb pollution is discussed by dry weight depending on the paper. To comparison with the Pb levels in those papers, Pb levels by dry weight in the current study are shown in Table 1.

Materials and Methods

Samples and Pb analysis were same with chapter 2. As for the preparation of Pb analysis, samples of 100–300 mg of soft tissues were dried for 24 hours at 50°C. Our preliminary experiment showed that 24h was enough for drying the samples.

Table 1. Sample information, assessment of Pb exposure and Pb concentration (dry and wet weight). m: male; f: female; ad.: adult; sub.: sub-adult; juv.: juvenile; ch.: chick; emb.: embryo; unk.: unknown; ND: non detectable

a) Liver and kidney specimens collected before the regulation in Hokkaido.

b) Liver and kidney specimens collected after the regulation in Hokkaido.

c) Liver and kidney specimens collected in Honshu and Shikoku.

d) Blood specimens collected in Hokkaido and Honshu

a)

Year	Species	Sex	Age	Liver (mg/kg)		Kidney (mg/kg)		Assessments
				dry wt	wet wt	dry wt	wet wt	
1998	Steller's sea eagle	unk.	unk.	0.94	0.29	0.04	0.01	high Pb exposure
1999	Steller's sea eagle	m	ad.	0.07	0.03	0.04	0.01	non-toxic
1999	Steller's sea eagle	f	sub.	74.85	27.90	34.30	9.88	Pb poisoning
1999	Steller's sea eagle	f	ad.	31.96	10.99	12.52	4.04	Pb poisoning
1999	White-tailed sea eagle	f	ad.	0.05	0.02	0.15	0.06	non-toxic

b)

Year	Species	Sex	Age	Liver (mg/kg)		Kidney (mg/kg)		Assessments
				dry wt	wet wt	dry wt	wet wt	
2008	Steller's sea eagle	m	ad.	48.61	16.55	16.52	4.93	Pb poisoning
2008	Steller's sea eagle	m	sub.	30.88	8.65	11.76	3.31	Pb poisoning
2009	Steller's sea eagle	m	juv.	0.08	0.03	0.08	0.03	non-toxic
2010	Steller's sea eagle	unk.	ad.	19.62	4.68	2.66	0.68	Pb poisoning
2010	Steller's sea eagle	m	sub.	70.81	23.81	17.08	4.19	Pb poisoning
2010	Steller's sea eagle	m	sub.	74.77	22.02	—	—	Pb poisoning
2011	Steller's sea eagle	m	ad.	0.50	0.22	0.50	0.16	high Pb exposure

2011	Steller’s sea eagle	unk.	ad.	0.19	0.05	—	—	non-toxic
2011	Steller’s sea eagle	unk.	ad.	121.06	36.56	26.37	8.20	Pb poisoning
2011	Steller’s sea eagle	f	ad.	112.49	32.36	37.56	11.02	Pb poisoning
2012	Steller’s sea eagle	f	juv.	0.03	0.01	0.06	0.01	non-toxic
2012	Steller’s sea eagle	unk.	juv.	0.12	0.04	0.16	0.05	non-toxic
2013	Steller’s sea eagle	f	juv.	0.07	0.02	0.07	0.02	non-toxic
2013	Steller’s sea eagle	f	juv.	0.03	0.01	—	—	non-toxic
2013	Steller’s sea eagle	f	ad.	0.04	0.01	0.09	0.03	non-toxic
2013	Steller’s sea eagle	f	juv.	0.04	0.01	0.36	0.15	non-toxic
2013	Steller’s sea eagle	f	sub.	65.17	17.16	12.78	3.00	Pb poisoning
2013	Steller’s sea eagle	m	ad.	0.16	0.05	0.18	0.07	non-toxic
2013	Steller’s sea eagle	m	sub.	0.13	0.04	0.07	0.03	non-toxic
2013	Steller’s sea eagle	f	ad.	54.83	15.94	22.35	5.34	Pb poisoning
2013	Steller’s sea eagle	f	ad.	75.92	22.89	—	—	Pb poisoning
2013	Steller’s sea eagle	unk.	juv.	9.70	2.99	17.56	4.91	Pb poisoning
2013	Steller’s sea eagle	unk.	juv.	10.96	3.71	—	—	Pb poisoning
2013	Steller’s sea eagle	m	ad.	0.21	0.06	0.38	0.10	non-toxic
2013	Steller’s sea eagle	m	ad.	98.14	36.29	52.62	14.72	Pb poisoning
2014	Steller’s sea eagle	m	ad.	0.10	0.03	0.10	0.02	non-toxic
2014	Steller’s sea eagle	m	ad.	0.01	0.00	0.01	0.00	non-toxic
2014	Steller’s sea eagle	unk.	juv.	0.01	0.00	—	—	non-toxic
2014	Steller’s sea eagle	f	juv.	0.02	0.01	0.01	0.00	non-toxic
2014	Steller’s sea eagle	unk.	juv.	0.01	0.00	0.01	0.00	non-toxic
2014	Steller’s sea eagle	unk.	ad.	0.09	0.02	0.09	0.02	non-toxic
2014	Steller’s sea eagle	unk.	ad.	45.19	11.55	—	—	Pb poisoning
2014	Steller’s sea eagle	m	ad.	0.50	0.17	0.33	0.08	non-toxic
2014	Steller’s sea eagle	unk.	juv.	4.06	1.08	0.81	0.19	high Pb exposure
2014	Steller’s sea eagle	unk.	ad.	64.68	15.32	10.78	2.10	Pb poisoning
2014	Steller’s sea eagle	f	ad.	0.03	0.01	0.02	0.00	non-toxic

2014	Steller’s sea eagle	unk.	sub.	0.07	0.02	0.06	0.01	non-toxic
2014	Steller’s sea eagle	f	ad.	58.39	18.33	18.27	4.65	Pb poisoning
2014	Steller’s sea eagle	unk.	juv.	ND	ND	0.00	0.00	non-toxic
2014	Steller’s sea eagle	f	ad.	0.04	0.01	0.04	0.01	non-toxic
2015	Steller’s sea eagle	m	sub.	75.27	20.46	36.88	8.01	Pb poisoning
2015	Steller’s sea eagle	f	ad.	124.95	32.94	46.37	8.47	Pb poisoning
2015	Steller’s sea eagle	m	juv.	0.04	0.01	0.04	0.01	non-toxic
2007	White-tailed sea eagle	f	sub.	0.33	0.11	1.05	0.34	non-toxic
2008	White-tailed sea eagle	f	ad.	67.25	23.30	58.40	14.00	Pb poisoning
2009	White-tailed sea eagle	m	ad.	163.62	53.74	32.63	8.48	Pb poisoning
2010	White-tailed sea eagle	f	sub.	69.92	22.05	4.82	1.32	Pb poisoning
2011	White-tailed sea eagle	unk.	ad.	0.14	0.06	0.28	0.08	non-toxic
2011	White-tailed sea eagle	f	juv.	35.42	10.83	32.44	9.12	Pb poisoning
2011	White-tailed sea eagle	m	sub.	0.10	0.02	0.10	0.02	non-toxic
2011	White-tailed sea eagle	m	juv.	0.05	0.02	0.10	0.03	non-toxic
2011	White-tailed sea eagle	m	sub.	0.04	0.02	0.08	0.02	non-toxic
2011	White-tailed sea eagle	f	sub.	95.51	31.56	34.07	9.06	Pb poisoning
2011	White-tailed sea eagle	m	ad.	0.11	0.03	0.06	0.02	non-toxic
2011	White-tailed sea eagle	m	unk.	0.13	0.05	0.13	0.04	non-toxic
2011	White-tailed sea eagle	unk.	sub.	0.19	0.07	—	—	non-toxic
2011	White-tailed sea eagle	unk.	ad.	0.07	0.02	—	—	non-toxic
2011	White-tailed sea eagle	f	sub.	18.88	6.72	20.72	6.22	Pb poisoning
2012	White-tailed sea eagle	unk.	sub.	0.82	0.26	2.22	0.48	high Pb exposure
2012	White-tailed sea eagle	f	sub.	0.08	0.03	0.39	0.10	non-toxic
2012	White-tailed sea eagle	unk.	sub.	0.07	0.03	0.16	0.05	non-toxic
2012	White-tailed sea eagle	f	ad.	0.98	0.34	4.55	1.14	high Pb exposure
2013	White-tailed sea eagle	unk.	juv.	34.07	10.25	27.96	6.87	Pb poisoning
2013	White-tailed sea eagle	unk.	juv.	0.02	0.00	0.09	0.02	non-toxic
2013	White-tailed sea eagle	unk.	ad.	0.05	0.02	0.05	0.01	non-toxic

2013	White-tailed sea eagle	m	sub.	175.12	56.41	36.95	8.39	Pb poisoning
2013	White-tailed sea eagle	unk.	juv.	0.03	0.01	0.08	0.02	non-toxic
2013	White-tailed sea eagle	unk.	sub.	0.02	0.00	—	—	non-toxic
2013	White-tailed sea eagle	m	ad.	0.08	0.03	0.12	0.03	non-toxic
2013	White-tailed sea eagle	unk.	juv.	0.01	0.00	0.08	0.02	non-toxic
2013	White-tailed sea eagle	unk.	juv.	ND	ND	—	—	non-toxic
2013	White-tailed sea eagle	unk.	juv.	0.76	0.17	0.88	0.15	non-toxic
2014	White-tailed sea eagle	unk	ad.	0.23	0.06	0.20	0.05	non-toxic
2014	White-tailed sea eagle	m	sub.	0.05	0.02	0.12	0.03	non-toxic
2014	White-tailed sea eagle	unk.	unk.	ND	ND	ND	ND	non-toxic
2014	White-tailed sea eagle	unk.	unk.	0.89	0.24	0.25	0.07	high Pb exposure
2014	White-tailed sea eagle	f	sub.	0.93	0.23	0.35	0.07	high Pb exposure
2014	White-tailed sea eagle	f	sub.	0.29	0.09	0.21	0.06	non-toxic
2014	White-tailed sea eagle	m	ad.	0.03	0.01	0.05	0.01	non-toxic
2014	White-tailed sea eagle	unk.	ad.	43.92	12.85	27.61	7.19	Pb poisoning
2014	White-tailed sea eagle	m	sub.	89.81	23.44	6.63	1.46	Pb poisoning
2014	White-tailed sea eagle	m	juv.	0.17	0.04	0.29	0.07	non-toxic
2014	White-tailed sea eagle	unk.	juv.	0.03	0.01	0.04	0.01	non-toxic
2014	White-tailed sea eagle	m	ad.	0.40	0.09	0.39	0.07	non-toxic
2014	White-tailed sea eagle	unk.	ad.	0.37	0.12	0.48	0.13	non-toxic
2014	White-tailed sea eagle	m	sub.	0.10	0.03	0.24	0.06	non-toxic
2015	White-tailed sea eagle	m	ad.	154.24	40.23	29.86	6.74	Pb poisoning
2015	White-tailed sea eagle	f	ad.	82.78	26.82	17.96	4.81	Pb poisoning
2015	White-tailed sea eagle	f	ad.	0.06	0.02	0.06	0.02	non-toxic
2015	White-tailed sea eagle	m	ad.	0.23	0.07	0.33	0.08	non-toxic
2015	White-tailed sea eagle	f	sub.	0.19	0.06	0.18	0.05	non-toxic
2015	White-tailed sea eagle	f	juv.	0.03	0.01	0.04	0.01	non-toxic
2015	White-tailed sea eagle	f	sub.	0.04	0.01	0.06	0.01	non-toxic
2015	White-tailed sea eagle	f	ad.	0.07	0.02	0.11	0.03	non-toxic

unk.	Blakiston’s fish owl	f	ad.	0.02	0.01	0.02	0.01	non-toxic
unk.	Blakiston’s fish owl	m	juv.	ND	ND	0.02	0.01	non-toxic
unk.	Blakiston’s fish owl	m	ad.	ND	ND	ND	ND	non-toxic
unk.	Blakiston’s fish owl	f	juv.	ND	ND	0.05	0.01	non-toxic
unk.	Blakiston’s fish owl	m	sub.	ND	ND	—	—	non-toxic
unk.	Blakiston’s fish owl	f	juv.	0.01	0.00	0.03	0.01	non-toxic
unk.	Blakiston’s fish owl	f	ch.	ND	ND	ND	ND	non-toxic
unk.	Blakiston’s fish owl	m	sub.	ND	ND	ND	ND	non-toxic
unk.	Blakiston’s fish owl	m	ad.	0.05	0.02	0.08	0.02	non-toxic
unk.	Blakiston’s fish owl	m	juv.	0.05	0.02	0.10	0.03	non-toxic
unk.	Blakiston’s fish owl	m	ad.	0.04	0.02	0.01	0.01	non-toxic
unk.	Blakiston’s fish owl	m	sub.	0.02	0.01	0.02	0.01	non-toxic
unk.	Blakiston’s fish owl	m	ad.	0.02	0.01	0.03	0.01	non-toxic
2014	Blakiston’s fish owl	f	juv.	0.13	0.04	0.10	0.02	non-toxic
2011	Mountain hawk eagle	unk.	juv.	0.02	0.01	0.02	0.00	non-toxic
2012	Mountain hawk eagle	unk.	juv.	ND	ND	0.02	0.00	non-toxic
2012	Mountain hawk eagle	m	sub.	0.02	0.01	0.06	0.02	non-toxic
2012	Mountain hawk eagle	unk.	ad.	0.26	0.09	0.18	0.06	non-toxic
2013	Mountain hawk eagle	unk.	sub.	ND	ND	0.00	0.00	non-toxic
2014	Mountain hawk eagle	unk.	ad.	0.13	0.04	0.04	0.01	non-toxic
2014	Mountain hawk eagle	f	ad.	0.07	0.02	0.12	0.04	non-toxic
unk.	Northern goshawk	unk.	juv.	0.12	0.03	0.05	0.01	non-toxic
unk.	Northern goshawk	unk.	ad.	0.01	0.00	0.00	0.00	non-toxic
unk.	Northern goshawk	unk.	sub.	0.07	0.02	0.09	0.03	non-toxic
unk.	Northern goshawk	m	juv.	0.08	0.02	0.05	0.02	non-toxic
unk.	Northern goshawk	f	ad.	0.02	0.01	0.05	0.01	non-toxic
2014	Northern goshawk	unk.	juv.	0.05	0.01	0.04	0.01	non-toxic
2012	Sparrowhawk	f	juv.	ND	ND	0.01	0.00	non-toxic
2015	Sparrowhawk	f	ad.	0.01	0.00	0.02	0.00	non-toxic

unk.	Peregrine falcon	unk.	juv.	0.45	0.12	1.11	0.32	non-toxic
2014	Black kite	unk.	sub.	0.05	0.01	0.07	0.01	non-toxic

c)

Year	Species	Sex	Age	Liver (mg/kg)		Kidney (mg/kg)		Assessments
				dry wt	wet wt	dry wt	wet wt	
1993	Golden eagle	m	sub.	0.08	0.03	—	—	non-toxic
1994	Golden eagle	unk.	unk.	0.07	0.03	—	—	non-toxic
1995	Golden eagle	m	sub.	6.65	2.31	—	—	Pb poisoning
1998	Golden eagle	unk.	juv.	0.39	0.14	—	—	non-toxic
1999	Golden eagle	unk.	ch.	0.10	0.03	—	—	non-toxic
2000	Golden eagle	unk.	unk.	0.29	0.07	—	—	non-toxic
2000	Golden eagle	unk.	unk.	0.14	0.02	—	—	non-toxic
2002	Golden eagle	unk.	emb.	0.16	0.04	—	—	non-toxic
2002	Golden eagle	f	emb.	0.02	0.01	—	—	non-toxic
2003	Golden eagle	unk.	juv.	0.38	0.12	—	—	non-toxic
2003	Golden eagle	f	unk.	0.12	0.03	—	—	non-toxic
2004	Golden eagle	m	unk.	0.17	0.05	—	—	non-toxic
2004	Golden eagle	m	ch.	0.87	0.27	—	—	high Pb exposure
1996	Northern goshawk	unk.	unk.	0.06	0.02	0.11	0.04	non-toxic
1999	Northern goshawk	unk.	unk.	1.05	0.35	0.83	0.21	high Pb exposure
2008	Northern goshawk	f	ad.	0.05	0.02	—	—	non-toxic
2009	Northern goshawk	f	ad.	0.05	0.02	—	—	non-toxic
2009	Northern goshawk	m	juv.	0.08	0.02	—	—	non-toxic
2009	Northern goshawk	f	ad.	0.04	0.01	—	—	non-toxic
2010	Northern goshawk	f	juv.	0.18	0.06	—	—	non-toxic
2011	Northern goshawk	unk.	unk.	0.04	0.01	—	—	non-toxic
2012	Northern goshawk	unk.	unk.	0.10	0.03	—	—	non-toxic
2007	Black kite	m	ad.	0.07	0.02	—	—	non-toxic

2008	Black kite	f	ad.	0.11	0.03	—	—	non-toxic
2008	Black kite	m	ad.	0.15	0.05	—	—	non-toxic
2008	Black kite	f	ad.	1.34	0.42	—	—	high Pb exposure
2008	Black kite	f	ad.	0.06	0.02	—	—	non-toxic
2010	Black kite	f	ad.	0.22	0.07	—	—	non-toxic
2010	Black kite	m	ad.	0.34	0.12	—	—	non-toxic
2012	Black kite	unk.	unk.	0.38	0.11	—	—	non-toxic
2013	Black kite	unk.	unk.	0.13	0.04	—	—	non-toxic
2012	Ural owl	unk.	unk.	0.05	0.02	—	—	non-toxic
2012	Ural owl	unk.	unk.	0.03	0.01	—	—	non-toxic
2012	Ural owl	unk.	unk.	0.02	0.01	—	—	non-toxic
2012	Ural owl	unk.	unk.	0.08	0.03	—	—	non-toxic
2010	Sparrowhawk	unk.	unk.	0.08	0.02	—	—	non-toxic
2013	Sparrowhawk	unk.	unk.	0.19	0.06	—	—	non-toxic
2013	Brown hawk owl	unk.	unk.	0.43	0.13	—	—	non-toxic
2013	Brown hawk owl	unk.	unk.	0.07	0.02	—	—	non-toxic
2006	Mountain hawk eagle	m	ad.	0.04	0.02	—	—	non-toxic
2006	Peregrine falcon	unk.	unk.	0.13	0.04	0.12	0.03	non-toxic
2011	Osprey	unk.	unk.	0.04	0.01	0.03	0.01	non-toxic
2012	Grey-faced buzzard	unk.	unk.	0.16	0.05	—	—	non-toxic
2012	Japanese sparrowhawk	unk.	unk.	0.01	0.00	—	—	non-toxic
2013	Common kestrel	unk.	unk.	0.06	0.01	—	—	non-toxic
2012	Sunda scops owl	unk.	unk.	0.03	0.01	—	—	non-toxic

d)

Year	Species	Place	Sex	Age	Blood (mg/kg, wet wt)	Assessments
2011	White-tailed sea eagle	Hokkaido	unk.	unk.	0.16	high Pb exposure
2013	Golden eagle	Honshu	unk.	unk.	0.14	high Pb exposure
2015	Mountain hawk eagle	Hokkaido	unk.	unk.	0.38	high Pb exposure

Note 2-2

Precision of the data measured by standard quadrupole model of ICP-MS using High Resolution multicollector ICP-MS (linked to the chapter 2)

Background and Objective

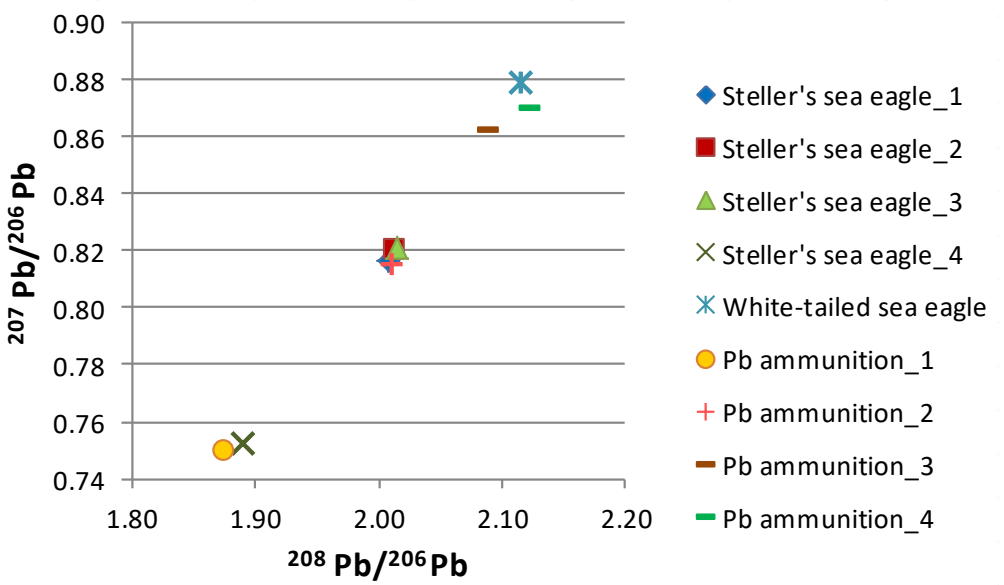
Our ICP-MS is the standard quadrupole model. This time, I confirmed the precision of our data using High Resolution Multicollector ICP-MS (NEPTUNE Plus) (Thermo Fisher Scientific, Inc, MA), that have high-precision for the measurement of isotope ratios.

Results and Discussion

Fig. 1 showed the results of Pb isotope ratios using our ICP-MS (a) and NEPTUNE Plus (b). Since I have about 200 samples, it was difficult to measure the all by NEPTUNE Plus, so I selected several samples which already showed distinctive isotope values by our initial measurement with ICP-MS. Both results with ICP-MS and NEPTUNE Plus showed the comparable values of Pb isotope ratios in each specimen. The high precision of NEPTUNE Plus was also shown in Table 1. Standard error of each individual ratio (2se) was very small in all specimens, and the isotope ratios of SRM 981 in our analysis were comparable with the certified value of SRM 981. I believe that these results indicate that the precision of our ICP-MS is acceptable.

Fig. 1.

(a) Pb isotope ratios using our ICP-MS.



(b) Pb isotope ratios using NEPTUNE Plus.

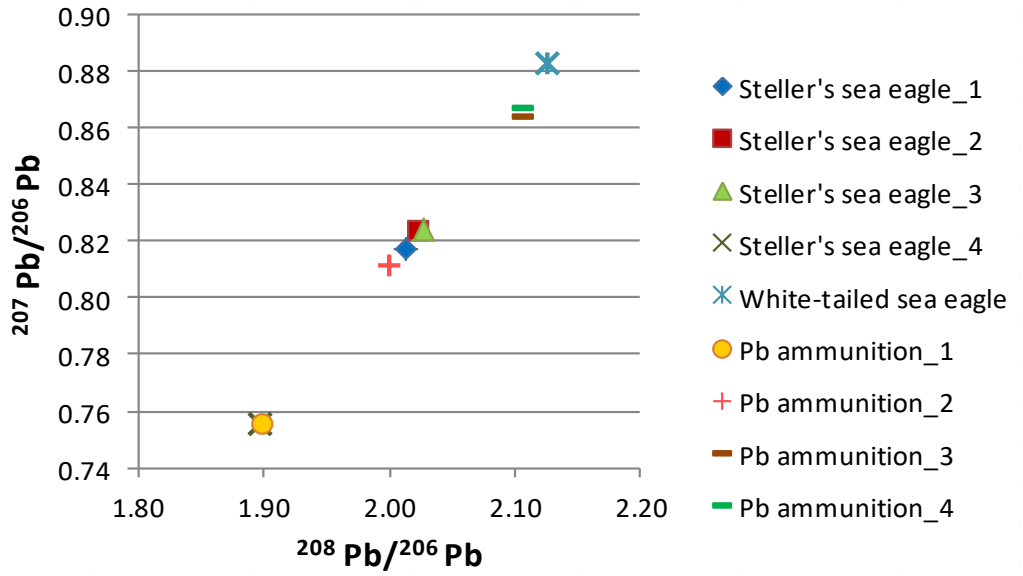


Table 1. Pb isotope ratios and the standard error of specimens by NEPTUNE Plus.

Sample	208Pb/206Pb	2se	207Pb/206Pb	2se
Steller's sea eagle_1	2.01304	0.00002	0.81719	0.00001
Steller's sea eagle_2	2.02460	0.00002	0.82332	0.00001
Steller's sea eagle_3	2.02698	0.00002	0.82436	0.00001
Steller's sea eagle_4	1.89808	0.00002	0.75505	0.00001
white-tailed sea eagle	2.12694	0.00001	0.88294	0.00000
Pb ammunition_1	1.89888	0.00036	0.75517	0.00021
Pb ammunition_2	2.00156	0.00002	0.81130	0.00001
Pb ammunition_3	2.10729	0.00002	0.86389	0.00001
Pb ammunition_4	2.10783	0.00002	0.86737	0.00001
SRM 981 (n = 5)	2.1664		0.9146	
Certified value of SRM 981	2.1677		0.9149	

CHAPTER 3

Current situation of lead exposure in birds in Japan (2015-2017); lead exposure is still occurring

Abstract

Birds belonging to a number of species have died as a result of lead (Pb) poisoning, including many Steller's sea eagles (*Haliaeetus pelagicus*) and white-tailed sea eagles (*Haliaeetus albicilla*) in Hokkaido, the northernmost island of Japan. To address this issue, the use of Pb ammunition was prohibited in Hokkaido more than 10 years ago. However, Pb poisoning is still being reported here and there are few regulations regarding the use of Pb ammunition in other parts of Japan, where it is reported that eagles have been exposed to Pb. Therefore, the objective of this study was to accurately determine the current level of Pb exposure in wild birds in Japan (June 2015–May 2017) and to identify the sources of Pb. It was found that Pb exposure is still occuring in raptors and waterbirds in various parts of Japan. In addition, Pb isotope ratio analysis showed that both Pb rifle bullets and Pb shot pellets cause Pb exposure in birds, and that endangered eagles are also exposed to Pb in Hokkaido due to the illegal use of Pb ammunition. It is concluded that changing to Pb-free ammunition, such as copper (Cu) rifle bullets, steel shot pellets, or bismuth shot pellets, is essential for the conservation of avian species in Japan.

Keywords

Pb exposure, Raptor, Waterbirds, Pb ammunition, Pb isotope ratios, Japan

Highlights

- Eagles and waterbirds in various parts of Japan are continuing to be exposed to Pb due to the accidental ingestion of Pb ammunition.
- In Hokkaido, endangered eagles are exposed to Pb through the illegal use of Pb ammunition.

1. Introduction

Birds belonging to a number of species have died as a result of lead (Pb) poisoning, including at least 33 raptors, 30 other terrestrial bird species, and waterbirds, in which Pb poisoning has been documented for many years (Arnemo et al., 2016; Pain et al., 2009). Raptors can experience secondary Pb poisoning from feeding on animals with Pb ammunition embedded in their tissues (Scheuhammer and Norris, 1996), while waterbirds commonly suffer from Pb poisoning through the ingestion of Pb shot pellets lying in lakes and marshes (Bellrose, 1959), which they mistake for food items or grit (Scheuhammer and Norris, 1996).

Pb is a broad-spectrum metabolic poison that produces toxic effects in a wide range of organs and tissues (Kendall et al., 1996), leading to high mortality. In addition, sub-lethal doses of Pb alter movement behaviors (Ecke et al., 2017), effect sperm quality and reproductive success (Vallverdú-Coll et al., 2016), and reduce the size of eggs and F1 generation hatchlings (Williams et al., 2017). The ecological significance of mortalities associated with Pb exposure must be viewed within the context of cumulative risks to avian populations (Kendall et al., 1996).

Although the use of Pb is banned from gasoline, paints, and various household items because of its toxicity, Pb ammunition is still widely used for hunting and shooting (Arnemo et al., 2016). Non-Pb shot, such as copper (Cu) rifle bullets or iron-tungsten-nickel shot pellets, have been developed (Brewer et al., 2003; Franson et al., 2012) and their efficacy has been demonstrated (Kanstrup et al., 2016). However, there are few nationally regulated bans on the use of Pb ammunition despite the overwhelming scientific evidence of the risks it poses and increasing policy imperatives (Arnemo et al., 2016).

There are several regulations for the use of Pb ammunition in Japan. In Hokkaido, the northernmost island of Japan, many Steller's sea eagles (*Haliaeetus pelagicus*) and white-tailed sea eagles (*Haliaeetus albicilla*) have died from Pb poisoning, leading to the implementation of a regulation to ban the use of Pb rifle bullets for hunting sika deer (*Cervus nipon*) in 2000 and prohibition of the use of Pb shot pellets for hunting sika deer since 2001. In addition, an extended ban was initiated in 2004, which prohibited the use of any type of Pb-containing ammunition for hunting large animal species. This regulation was enforced in 2014, prohibiting the possession of Pb rifle bullets, slugs, or large shot pellets during hunting (prior to this regulation, it was not illegal to carry Pb ammunition when hunting but hunters were punished if they were found to have used such ammunition). However, although more than 10 years have passed since the legislation regarding Pb ammunition was introduced, Pb poisoning is still being reported in Hokkaido (Ishii et al., 2017).

There are few regulations regarding the use of Pb ammunition in other parts of Japan, resulting in eagles being exposed to Pb. In addition, Pb poisoning has been reported in waterbirds (Honda et al., 1990; Ochiai et al., 1993), with several Pb-poisoned swans having been detected in Honshu in 2016 (Authors, unpublished data).

The relative abundances of the naturally occurring isotopes of Pb (^{204}Pb, ^{206}Pb, ^{207}Pb, and ^{208}Pb) vary among industrial materials, allowing the isotope ratios in organisms to be used to determine the source of Pb exposure, as previously studied in raptors (Church et al., 2006). Therefore, the objective of this study was to accurately determine the current levels of Pb exposure in wild birds in Japan (June 2015– May 2017) and to identify the source of Pb by analyzing the Pb isotope ratios.

2. Materials and Methods

2.1. Sampling

Samples of birds that died in the wild or at medical centers for wild birds in Japan were collected for analysis of their Pb concentrations and Pb isotope ratios. Liver specimens were collected from June 2015 to May 2017 from the following locations and species: from Hokkaido, white-tailed sea eagle (n = 27), Steller's sea eagle (n = 21), Blakiston's fish owl (*Ketupa blakistoni*) (n = 6), mountain hawk eagle (*Spizaetus nipalensis*) (n = 3), Eurasian hobby (*Falco subbuteo*) (n = 2), northern goshawk (*Accipiter gentilis*) (n = 1), peregrine falcon (*Falco peregrinus*) (n = 1), sparrow hawk (*Accipiter nisus*) (n =1), ural owl (*Strix uralensis japonica*) (n = 1), jungle crow (*Corvus macrorhynchos*) (n = 2), and whooper swan (*Cygnus cygnus*) (n = 1); and from Honshu, mountain hawk eagle (n = 1), northern goshawk (n = 1), peregrine falcon (n = 1), whooper swan (n = 2), and greater white-fronted goose (*Anser albifrons*) (n = 2). Swans that had died in one lake in Honshu (see Introduction) were not included in the analysis to exclude any potential sampling bias around the ratio of Pb exposure in swans. The specimens were transported to the Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan, where they were stored at –20°C until analysis. The ages of raptors were estimated from their morphological characteristics, such as the development of the gonads and feathers, the color of their feathers and iris, and the molting condition.

Four shot pellets from the stomach of a whooper swan and a fragment of Pb ammunition from the stomach of a Steller's sea eagle were also collected to analyze the Pb isotope ratios in Pb ammunition.

2.2. Pb concentration and stable isotope analysis

Pb concentrations were analyzed according to the method of Yabe et al. (2015). Samples of 100–300 mg of soft tissues were digested with 5 mL of 30% nitric acid (Kanto Chemical Corporation, Tokyo, Japan) and 1 mL of 30% hydrogen peroxide (Kanto Chemical Corporation) in a microwave digestion system (Speedwave Two; Berghof, Eningen, Germany), after which the volume was made up to 10 mL by adding 2% nitric acid. Digestion was performed under the following conditions: 180°C for 15 minutes, 200°C for 20 minutes, and 100°C for 20 minutes. The Pb concentration and isotope ratios were then measured using an inductively coupled plasma–mass spectrometer (ICP-MS) (7700 series; Agilent Technology, Tokyo, Japan) (see Table S1 for detailed analytical conditions), which was calibrated using ICP-MS Calibration Standards (Agilent Technology) to establish standard curves before analysis. Standard solutions (0, 10, 50, 100, 250, and 500 μg/L) were prepared with 2% nitric acid and the standard curves had r^2 values of 0.998. All chemicals and standard stock solutions were of analytical reagent grade (Wako Pure Chemicals Industries, Osaka, Japan). Distilled and deionized water was used (Milli-Q; Merck Millipore, Billerica, MA, USA), and an analytical quality control was performed using DOLT-4 (dogfish liver) and DORM-3 (fish protein) certified reference materials (National Research Council of Canada, Ottawa, Canada), which were shown to have good recoveries (95–105%) through replicate analysis. Thallium (^{205}Tl) was used as an internal standard for the Pb concentration analysis but not for the stable isotope ratio analysis. The limit of detection for Pb was 0.01 μg/kg.

The Pb isotope ratios were analyzed according to the method of Nakata et al. (2015). Dissolved samples were diluted to a Pb concentration of <25 μg/L with 2% nitric acid. NIST SRM 981 (National Institute of Standards and Technology, Gaithersburg, MD, USA) was used as a standard reference material for the external standardization of Pb

isotopes, which showed that the relative standard deviation (RSD) of the ratios was <0.5% for both $^{207}Pb/^{206}Pb$ and $^{208}Pb/^{207}Pb$. Therefore, any samples for which the RSD value exceeded 0.5% were excluded from the analysis. Blanks and standard solutions were measured every 10 samples for recalibration.

2.3. Statistical analysis

Differences in the Pb concentrations among sexes and ages of Steller's sea eagles and white-tailed sea eagles were analyzed using the Mann–Whitney U test (for species and sex) or the Steel-Dwass test (for age). All statistical analyses were performed in JMP Pro 11 (SAS Institute, Cary, NC, USA) with a significance level of $p < 0.05$.

3. Results

Various thresholds for Pb toxicity in birds have been reported in the literature (Fisher et al., 2006; Kendall et al., 1996; Kim et al., 1999; Kurosawa, 2000; Saito, 2009). The background level of Pb in the liver is generally <2 mg/kg wet weight (6–7 mg/kg dry weight) or <1 mg/kg wet weight (3 mg/kg dry weight) in raptors (Pain and Amiardtriquet, 1993). Some authors have reported that a liver Pb concentration of >6 mg/kg dry weight in raptors indicates an abnormally high exposure to Pb, while a concentration of >20 mg/kg dry weight indicates acute exposure and absorption, resulting in Pb poisoning (Pain and Amiardtriquet, 1993; Pain et al., 1995). The following categories are used in Japan based on the hepatic Pb concentration in wet weight: <0.2 mg/kg, normal range; 0.2–2 mg/kg, high level of Pb exposure; and >2 mg/kg, Pb poisoning (Saito, 2009).

In total, 3 out of 27 white-tailed sea eagles, 6 out of 21 Steller's sea eagles, and 2 out of 3 whooper swans exceeded the background level of Pb poisoning (i.e., had >2 mg/kg wet weight of Pb in the liver). In addition, one white-tailed sea eagle, two Steller's sea eagles, one mountain hawk eagle, one Eurasian hobby, and one greater white-fronted goose showed high exposure of Pb (0.2–2 mg/kg) (Table 1). Therefore, it is clear that despite the use of Pb ammunition being prohibited in Hokkaido and limited parts of Honshu in 2014, raptors and waterbirds were continuing to be poisoned by Pb. It was also found that female white-tailed sea eagles accumulated higher concentrations of Pb than males ($P = 0.03$; individuals of unknown sex were removed from the analysis), but there were no significant differences between the sexes or age groups for any other species.

The following Pb isotope ratios ($^{208}Pb/^{206}Pb$, $^{207}Pb/^{206}Pb$) were reported for various types of Pb ammunition obtained from shops in Japan: rifle bullets, 1.90–2.10 and 0.75–0.88; shot pellets, 2.07–2.14 and 0.85–0.87; and fishing sinkers, 2.08–2.20 and

0.84–0.90. These materials had been purchased or collected from the carcasses of birds up until 2001. We confirmed that the Pb isotope ratios of Pb rifle bullets, shot pellets, and fishing sinkers used in 2015 by hunters and fishers in Japan were comparable to these values.

The Pb isotope ratios indicated that almost all of the white-tailed sea eagles and Steller's sea eagles examined were exposed to Pb from Pb rifle bullets, while several sea eagles had ingested Pb shot pellets (Figure 1). In addition, one mountain hawk eagle contained Pb from a Pb rifle bullet, while Eurasian hobbies, whooper swans, and one greater white-fronted goose had ingested Pb shot pellets (see Figure S1 for the results of the $^{208}Pb/^{206}Pb$ and $^{207}Pb/^{206}Pb$ isotope analysis). One fragment of Pb ammunition that was extracted from the stomach of a Steller's sea eagle had the same isotope ratios as occurred in the liver of the same individual, while four shot pellets from one whooper swan had different Pb isotope ratios, with the Pb isotope ratios in the swan itself representing the median value (Table S3).

4. Discussion

In this study, it was demonstrated that Pb exposure has occured in various species of raptors and waterbirds in many parts of Japan. In addition, it was clear that this Pb exposure resulted from both Pb rifle bullets and Pb shot pellets, with the latter being the main cause in waterbirds. It is possible that the waterbirds were exposed to Pb in fishing tackle, which has the same Pb isotope ratios as shot pellets. However, since Pb shot pellets were found in the stomachs of swans, these are evidently one of the sources of Pb exposure in waterbirds in Japan.

Endangered eagles were poisoned by Pb in Hokkaido, despite the use of Pb ammunition having been prohibited for more than 10 years. The main cause of Pb exposure in sea eagles was found to be Pb rifle bullets, altough these eagles were also exposed to Pb shot pellets. These results demonstrate that the regulation around the use of Pb ammunition is not working well. Only limited numbers of birds were obtained from Honshu, but it was clear that waterbirds here were exposed to Pb from Pb shot pellets. In addition, the finding that one swan contained four different Pb isotope ratios in four Pb shot pellets indicates that several types of Pb shot pellets had been used in the same area.

White-tailed sea eagles showed sex differences in Pb accumulation, with females accumulating higher concentrations of Pb than males. Similarly, it has previously been reported that female common grackles (*Quiscalus quiscula*) accumulate higher concentrations of Pb than males (Beyer et al., 1988). However, no significant differences between the sexes were found in a previous study of sea eagles in Hokkaido (Ishii et al., 2017). Therefore, since the sex of many eagles was unknown in the present study, further data are required to make a more accurate comparison.

This study showed that an improvement to the regulation to prohibit the use of

Pb ammunition across all parts of Japan is required to conserve avian species. It has been demonstrated that Pb-free ammunition, such as copper (Cu) rifle bullets, steel shot pellets, or bismuth shot pellets, have the same or even greater effectiveness as Pb ammunition for hunting, and are much less toxic to birds (Kanstrup et al., 2016; Scheuhammer and Norris, 1996). In Denmark, it has been reported that Cu ammunition within the tested range of bullet calibers can be recommended as an effective alternative to lead-core bullets (Kanstrup et al., 2016). In addition, in the case of rifle bullets, Pb rifle bullets break into pieces within the bodies of hunted animals, whereas Cu rifle bullets do not, causing less damage to the hunted animals. Therefore, it is also have some benefits for game animals in terms of lethality and animal welfare (Kanstrup et al., 2016). Therefore, the provision of accurate information about Pb exposure in birds and the efficacy of non-Pb ammunition will be important to improve the current situation in Japan.

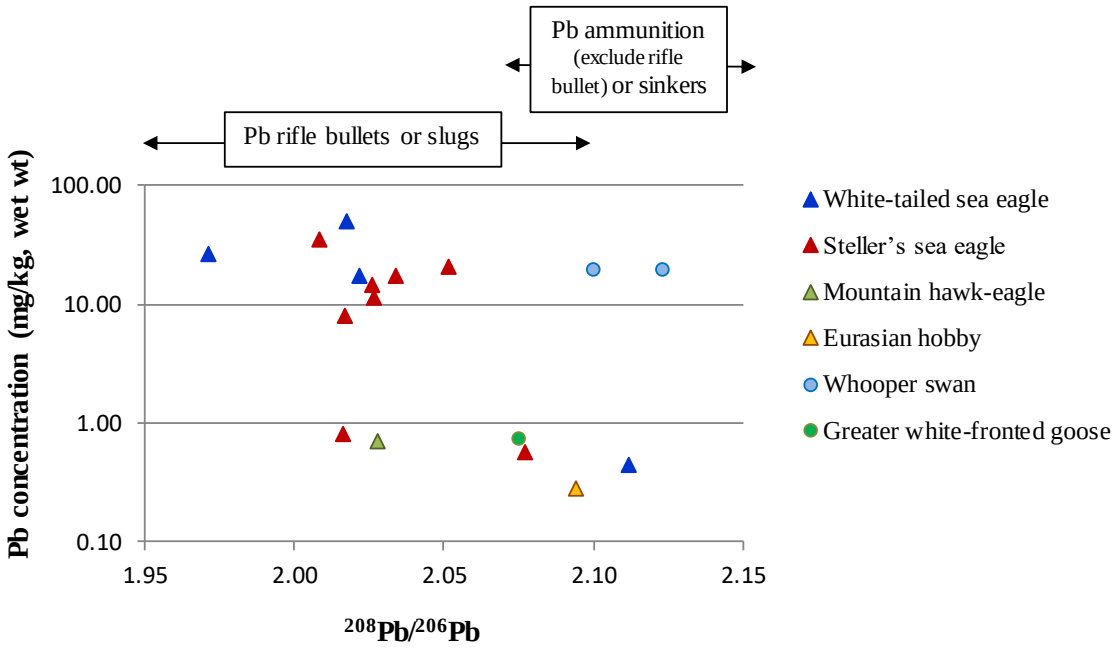
5. Conclusions

The present study showed that Pb exposure continues to occur in eagles and waterbirds in various parts of Japan due to the accidental ingestion of Pb ammunition. In Hokkaido, endangered eagles are exposed to Pb, with Pb isotope ratios indicating that these eagles are poisoned by Pb ammunition that is used illegally. Pb exposure in birds was also confirmed in other parts of Japan, where there are only limited regulations on the use of Pb ammunition. Based on these results, it is clear that non-Pb ammunition needs to be used for hunting to reduce Pb exposure and conserve wild birds in Japan.

Table 1. Hepatic Pb levels (mg/kg, wet weight, range) and the assesments of Pb exposure in birds.

Species	Sample size	Pb concentration in liver	Assessments		
		mg/kg, wet wt, median, (range)	Pb poisoning (> 2.0 mg.kg)	High Pb exposure (0.2 - 2.0 mg/kg)	Non toxic (< 0.2 mg/kg)
White-tailed sea eagle	27	0.10 (0.03 - 50.79)	3	1	23
Steller's sea eagle	21	0.10 (0.05 - 35.36)	6	2	13
Blakiston's fish-owl	6	0.05 (0.02 - 0.07)	0	0	6
Mountain hawk-eagle	4	0.11 (0.05 - 0.70)	0	1	3
Eurasian hobby	2	0.19 (0.09 - 0.28)	0	1	1
Northern goshawk	2	0.15 (0.14 - 0.16)	0	0	2
Peregrine falcon	2	0.09 (0.09)	0	0	2
Sparrowhawk	1	0.06	0	0	1
Ural owl	1	0.14	0	0	1
Jungle crow	2	0.08 (0.06 - 0.11)	0	0	2
Whooper swan	3	19.56 (0.18 - 19.58)	2	0	1
Greater white-fronted goose	2	0.41 (0.10 - 0.72)	0	1	1

Fig. 1. Comparison of Pb levels and Pb isotope ratio ($^{208}Pb/^{206}Pb$, RSD < 0.5) in the liver of birds that had high Pb concentration (> 0.2 mg/kg, wet weight).



References

Arnemo, J.M., Andersen, O., Stokke, S., Thomas, V.G., Krone, O., Pain, D.J., Mateo, R., 2016. Health and environmental risks from lead-based ammunition: science versus socio-politics. Ecohealth 13, 618–622.

Bellrose, F.C., 1959. Lead poisoning as a mortality factor in waterfowl populations. Illinois Nat. Hist. Surv. Bull. 27, 235-288

Beyer, W.N., Spann, J.W., Sileo, L., Franson, J.C., 1988. Lead poisoning in six captive avian species. Arch. Environ. Contam. Toxicol. 17, 121–130.

Brewer, L., Fairbrother, A., Clark, J., Amick, D., 2003. Acute toxicity of lead, steel, and an iron-tungsten-nickel shot to mallard ducks (*Anas platyrhynchos*). J. Wildl. Dis. 39, 638–648.

Church, M.E., Gwiazda, R., Risebrough, R.W., Sorenson, K., Chamberlain, C.P., Farry, S., Heinrich, W., Rideout, B.A., Smith, D.R., 2006. Ammunition is the principal source of lead accumulated by California condors re-introduced to the wild. Environ. Sci. Technol. 40, 6143–6150.

Ecke, F., Singh, N.J., Arnemo, J.M., Bignert, A., Helander, B., Berglund, Å.M.M., Borg, H., Bröjer, C., Holm, K., Lanzone, M., 2017. Sublethal Lead Exposure Alters Movement Behavior in Free-Ranging Golden Eagles. Environ. Sci. Technol. 51, 5729–5736.

Fisher, I.J., Pain, D.J., Thomas, V.G., 2006. A review of lead poisoning from ammunition sources in terrestrial birds. Biol. Conserv. 131, 421–432. doi:http://dx.doi.org/10.1016/j.biocon.2006.02.018

Franson, J.C., Lahner, L.L., Meteyer, C.U., Rattner, B.A., 2012. Copper pellets simulating oral exposure to copper ammunition: absence of toxicity in American

kestrels (*Falco sparverius*). Arch. Environ. Contam. Toxicol. 62, 145–153.

Honda, K., Lee, D.P., Tatsukawa, R., 1990. Lead poisoning in swans in Japan. Environ. Pollut. 65, 209–218.

Ishii, C., Nakayama, S.M.M., Ikenaka, Y., Nakata, H., Saito, K., Watanabe, Y., Mizukawa, H., Tanabe, S., Nomiyama, K., Hayashi, T., 2017. Lead exposure in raptors from Japan and source identification using Pb stable isotope ratios. Chemosphere 186, 367–373.

Kanstrup, N., Balsby, T.J.S., Thomas, V.G., 2016. Efficacy of non-lead rifle ammunition for hunting in Denmark. Eur. J. Wildl. Res. 62, 333–340.

Kendall, R.J., Lacker, T.E., Bunck, C., Daniel, B., Driver, C., Grue, C.E., Leighton, F., Stansley, W., Watanabe, P.G., Whitworth, M., 1996. An ecological risk assessment of lead shot exposure in non-waterfowl avian species: Upland game birds and raptors. Environ. Toxicol. Chem. 15, 4–20. doi:10.1002/etc.5620150103

Kim, E.-Y., Goto, R., Iwata, H., Masuda, Y., Tanabe, S., Fujita, S., 1999. Preliminary survey of lead poisoning of Steller's sea eagle (*Haliaeetus pelagicus*) and white-tailed sea eagle (*Haliaeetus albicilla*) in Hokkaido, Japan. Environ. Toxicol. Chem. 18, 448–451. doi:10.1002/etc.5620180312

Kurosawa, N., 2000. Lead poisoning in Steller's sea eagles and white-tailed sea eagles, in: First Symposium on Stellar's and White-Tailed Sea Eagles in East Asia. Wild Bird Society of Japan, Tokyo. pp. 107–109.

Nakata, H., Nakayama, S.M.M., Ikenaka, Y., Mizukawa, H., Ishii, C., Yohannes, Y.B., Konnai, S., Darwish, W.S., Ishizuka, M., 2015. Metal extent in blood of livestock from Dandora dumping site, Kenya: Source identification of Pb

exposure by stable isotope analysis. Environ. Pollut. 205, 8–15.

Ochiai, K., Hoshiko, K., Jin, K., Tsuzuki, T., Itakura, C., 1993. A survey of lead poisoning in wild waterfowl in Japan. J. Wildl. Dis. 29, 349–352.

Pain, D.J., Amiardtriquet, C., 1993. Lead Poisoning of Raptors in France and Elsewhere. Ecotoxicol. Environ. Saf. 25, 183–192. doi:http://dx.doi.org/10.1006/eesa.1993.1017

Pain, D.J., Fisher, I.J., Thomas, V.G., 2009. A global update of lead poisoning in terrestrial birds from ammunition sources. Ingestion lead from spent Ammunit. Implic. Wildl. humans 99–118.

Pain, D.J., Sears, J., Newton, I., 1995. Lead concentrations in birds of prey in Britain. Environ. Pollut. 87, 173–180. doi:http://dx.doi.org/10.1016/0269-7491(94)P2604-8

Saito, K., 2009. Lead poisoning of Steller's Sea-Eagle (*Haliaeetus pelagicus*) and White tailed Eagle (*Haliaeetus albicilla*) caused by the ingestion of lead bullets and slugs. Hokkaido Japan. RT Watson, M. Fuller, M. Pokras, WG Hunt (Eds.). Ingestion Lead from Spent Ammunit. Implic. Wildl. Humans. Peregrine Fund, Boise, Idaho, USA.

Scheuhammer, A.M., Norris, S.L., 1996. The ecotoxicology of lead shot and lead fishing weights. Ecotoxicology 5, 279–295.

Vallverdú-Coll, N., Mougeot, F., Ortiz-Santaliestra, M.E., Castaño, C., Santiago-Moreno, J., Mateo, R., 2016. Effects of Lead Exposure on Sperm Quality and Reproductive Success in an Avian Model. Environ. Sci. Technol. 50, 12484–12492.

Williams, R.J., Tannenbaum, L. V, Williams, S.M., Holladay, S.D., Tuckfield, R.C.,

Sharma, A., Humphrey, D.J., Gogal, R.M., 2017. Ingestion of a Single 2.3 mm Lead Pellet by Laying Roller Pigeon Hens Reduces Egg Size and Adversely Affects F1 Generation Hatchlings. Arch. Environ. Contam. Toxicol. 1–9.

Yabe, J., Nakayama, S.M.M., Ikenaka, Y., Yohannes, Y.B., Bortey-Sam, N., Oroszlany, B., Muzandu, K., Choongo, K., Kabalo, A.N., Ntapisha, J., Mweene, A., Umemura, T., Ishizuka, M., 2015. Lead poisoning in children from townships in the vicinity of a lead-zinc mine in Kabwe, Zambia. Chemosphere 119, 941–947. doi:10.1016/j.chemosphere.2014.09.028

Supplementary data

Table S1. Detailed analytical conditions of ICP-MS (7700 series, Agilent technologies) for analysis of Pb concentration (a) and Pb isotope ratios (b).

Table S2. Sample information and Pb concentration (wet weight), assessments of Pb exposure and Pb isotope ratios ($^{208}Pb/^{206}Pb$ and $^{207}Pb/^{206}Pb$).

Table S3. Pb isotope ratios in liver and ammunition from the stomach of Steller's sea eagle and whooper swan

Fig. S1. Pb isotope ratios ($^{208}Pb/^{206}Pb$, RSD < 0.5) in the liver of birds that had high Pb concentrations (> 0.2 mg/kg, wet weight).

Table S1. Detailed analytical conditions of ICP-MS (7700 series, Agilent technologies) for analysis of Pb concentration (a) and Pb isotope ratios (b).

(a)

Parameter	Value
RF Power	1500 W
Argon gas pressure	600 kPa
Cell gas (Helium)	100 kPa
Peak pattern	1
Replicates	3
Sweeps/replicate	100
Stabilization time	30 s

(b)

Parameter	Value
RF Power	1500 W
Argon gas pressure	600 kPa
Cell gas (Helium)	100 kPa
Peak pattern	3
Replicates	10
Sweeps/replicate	1000
Integration time/mass	9.00 s
Stabilization time	40 s

Table S2. Sample information and Pb concentration (wet weight), assessments of Pb exposure and Pb isotope ratios ($^{208}Pb/^{206}Pb$ and $^{207}Pb/^{206}Pb$).

m: male; f: female; ad.: adult; sub.: sub-adult; juv.: juvenile; ch.: chick; emb.: embryo; unk.: unknown

Year	Species	Places	Sex	Age	Pb level (mg/kg) wet wt.	Assessments	Pb isotope ratios	
							208/206 Pb	207/206 Pb
2015	White-tailed sea eagle	Hokkaido	unk.	unk.	50.79	Pb poisoning	2.02	0.82
2015	White-tailed sea eagle	Hokkaido	unk.	unk.	0.09	Non toxic		
2015	White-tailed sea eagle	Hokkaido	f	sub.	0.15	Non toxic	2.04	0.83
2015	White-tailed sea eagle	Hokkaido	f	juv.	0.17	Non toxic	2.05	0.83
2016	White-tailed sea eagle	Hokkaido	unk.	unk.	0.07	Non toxic		
2016	White-tailed sea eagle	Hokkaido	f	ad.	0.12	Non toxic		
2016	White-tailed sea eagle	Hokkaido	f	juv.	0.15	Non toxic		
2016	White-tailed sea eagle	Hokkaido	f	juv.	0.12	Non toxic		
2016	White-tailed sea eagle	Hokkaido	f	juv.	0.07	Non toxic		
2016	White-tailed sea eagle	Hokkaido	unk.	unk.	0.16	Non toxic		
2016	White-tailed sea eagle	Hokkaido	unk.	unk.	0.03	Non toxic		
2016	White-tailed sea eagle	Hokkaido	f	ad.	0.44	High Pb exposure	2.11	0.87
2017	White-tailed sea eagle	Hokkaido	unk.	unk.	17.54	Pb poisoning	2.02	0.82
2017	White-tailed sea eagle	Hokkaido	f	sub.	0.07	Non toxic		
2017	White-tailed sea eagle	Hokkaido	m	sub.	0.08	Non toxic		
2017	White-tailed sea eagle	Hokkaido	unk.	unk.	0.16	Non toxic		

2017	White-tailed sea eagle	Hokkaido	m	sub.	0.10	Non toxic		
2017	White-tailed sea eagle	Hokkaido	unk.	sub.	0.06	Non toxic		
2017	White-tailed sea eagle	Hokkaido	unk.	unk.	0.16	Non toxic	2.05	0.83
2017	White-tailed sea eagle	Hokkaido	unk.	unk.	0.05	Non toxic	2.03	0.83
2017	White-tailed sea eagle	Hokkaido	unk.	unk.	0.03	Non toxic		
2017	White-tailed sea eagle	Hokkaido	f	ad.	26.29	Pb poisoning	1.97	0.80
2017	White-tailed sea eagle	Hokkaido	m	sub.	0.03	Non toxic	2.05	0.84
2017	White-tailed sea eagle	Hokkaido	m	ad.	0.09	Non toxic		
2017	White-tailed sea eagle	Hokkaido	m	ad.	0.07	Non toxic		
2017	White-tailed sea eagle	Hokkaido	f	ad.	0.13	Non toxic		
2017	White-tailed sea eagle	Hokkaido	m	ad.	0.08	Non toxic	2.03	0.83
2016	Steller’s sea eagle	Hokkaido	unk.	unk.	11.51	Pb poisoning	2.03	0.83
2016	Steller’s sea eagle	Hokkaido	f	unk.	0.08	Non toxic		
2016	Steller’s sea eagle	Hokkaido	unk.	unk.	14.80	Pb poisoning	2.03	0.82
2016	Steller’s sea eagle	Hokkaido	unk.	ad.	17.67	Pb poisoning	2.03	0.83
2016	Steller’s sea eagle	Hokkaido	unk.	unk.	0.10	Non toxic	2.09	0.85
2016	Steller’s sea eagle	Hokkaido	f	ad.	7.94	Pb poisoning	2.02	0.82
2016	Steller’s sea eagle	Hokkaido	f	ad.	0.10	Non toxic		
2016	Steller’s sea eagle	Hokkaido	unk.	unk.	0.05	Non toxic		
2016	Steller’s sea eagle	Hokkaido	unk.	unk.	0.07	Non toxic		
2017	Steller’s sea eagle	Hokkaido	m	ad.	0.07	Non toxic		
2017	Steller’s sea eagle	Hokkaido	f	ad.	0.07	Non toxic		
2017	Steller’s sea eagle	Hokkaido	unk.	unk.	20.45	Pb poisoning	2.05	0.84

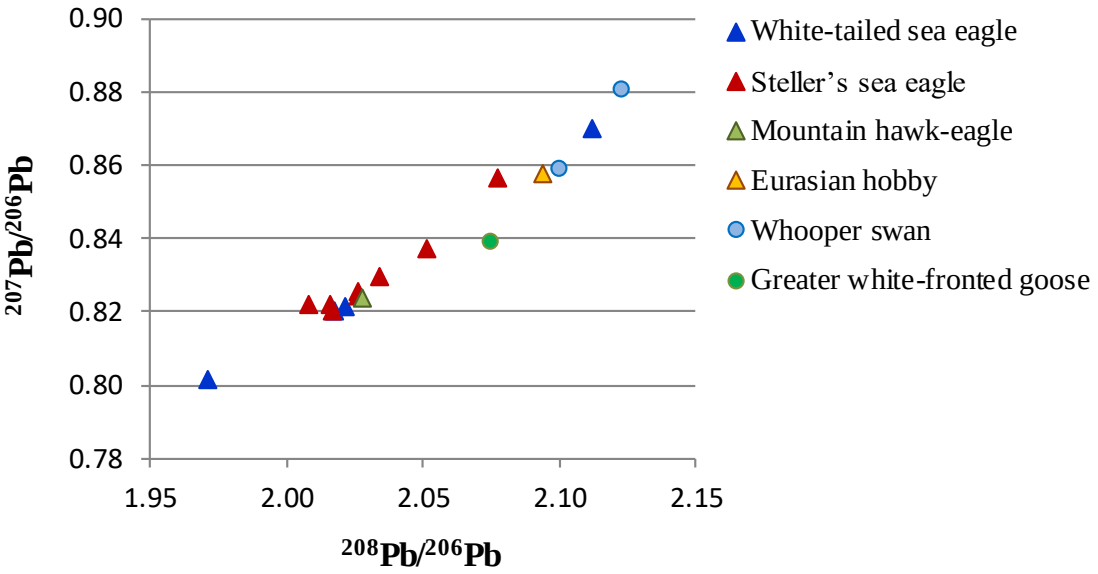
2017	Steller’s sea eagle	Hokkaido	unk.	unk.	0.11	Non toxic		
2017	Steller’s sea eagle	Hokkaido	m	ad.	0.11	Non toxic		
2017	Steller’s sea eagle	Hokkaido	f	ad.	0.57	High Pb exposure	2.08	0.86
2017	Steller’s sea eagle	Hokkaido	m	ad.	35.36	Pb poisoning	2.01	0.82
2017	Steller’s sea eagle	Hokkaido	f	sub.	0.80	High Pb exposure	2.02	0.82
2017	Steller’s sea eagle	Hokkaido	f	ad.	0.07	Non toxic		
2017	Steller’s sea eagle	Hokkaido	f	sub.	0.05	Non toxic	2.05	0.83
2017	Steller’s sea eagle	Hokkaido	unk.	unk.	0.05	Non toxic	2.04	0.83
2017	Steller’s sea eagle	Hokkaido	m	ad.	0.05	Non toxic		
2015	Blakiston's fish-owl	Hokkaido	f	unk.	0.04	Non toxic		
2015	Blakiston's fish-owl	Hokkaido	f	unk.	0.06	Non toxic		
2016	Blakiston's fish-owl	Hokkaido	m	sub.	0.02	Non toxic		
2017	Blakiston's fish-owl	Hokkaido	unk.	ch.	0.06	Non toxic	2.03	0.83
2017	Blakiston's fish-owl	Hokkaido	f	juv.	0.07	Non toxic		
2016	Blakiston's fish-owl	Hokkaido	m	sub.	0.02	Non toxic		
2016	Mountain hawk-eagle	Hokkaido	unk.	unk.	0.70	High Pb exposure	2.03	0.82
2017	Mountain hawk-eagle	Hokkaido	unk.	unk.	0.10	Non toxic		
2015	Mountain hawk-eagle	Honshu	unk.	unk.	0.05	Non toxic		
2017	Mountain hawk-eagle	Hokkaido	m	sub.	0.12	Non toxic	1.99	0.81
2015	Eurasian hobby	Hokkaido	unk.	unk.	0.28	High Pb exposure	2.09	0.86
2016	Eurasian hobby	Hokkaido	m	juv.	0.09	Non toxic		
2015	Northern goshawk	Hokkaido	f	unk.	0.14	Non toxic	2.04	0.82
2016	Northern goshawk	Honshu	unk.	unk.	0.16	Non toxic		
2016	Peregrine falcon	Hokkaido	f	juv.	0.09	Non toxic		

2015	Peregrine falcon	Honshu	unk.	unk.	0.09	Non toxic		
2016	Sparrowhawk	Hokkaido	f	sub.	0.06	Non toxic		
2016	Ural owl	Hokkaido	f	ad.	0.14	Non toxic		
2015	Jungle crow	Hokkaido	m	unk.	0.06	Non toxic		
2015	Jungle crow	Hokkaido	m	unk.	0.11	Non toxic		
2016	Whooper swan	Honshu	unk.	unk.	0.18	Non toxic	2.07	0.84
2016	Whooper swan	Honshu	unk.	unk.	19.58	Pb poisoning	2.12	0.88
2016	Whooper swan	Hokkaido	m	unk.	19.56	Pb poisoning	2.10	0.86
2016	Greater white-fronted goose	Honshu	unk.	unk.	0.10	Non toxic	2.06	0.83
2016	Greater white-fronted goose	Honshu	unk.	unk.	0.72	High Pb exposure	2.08	0.84

Table S3. Pb isotope ratios in liver and ammunition from the stomach of Steller's sea eagle and whooper swan

	Sample	Pb isotope ratios	
		208/206 Pb	207/206 Pb
Steller's sea eagle	Liver	2.05	0.84
	Ammunition from the stomach	2.05	0.84
Whooper swan	Liver	2.12	0.88
	Ammunition from the stomach_1	2.16	0.90
	Ammunition from the stomach_2	2.11	0.87
	Ammunition from the stomach_3	2.10	0.86
	Ammunition from the stomach_4	2.10	0.86

Fig. S1. Pb isotope ratios ($^{208}Pb/^{206}Pb$, RSD < 0.5) in the liver of birds that had high Pb concentrations (> 0.2 mg/kg, wet weight).



Note 3-1

Pb concentration in liver by dry weight (linked to the chapter 3)

Background and Objective

As for the chapter 3, Pb concentrations by dry weight were not shown. However, Pb pollution is discussed by dry weight depending on the paper. To comparison with the Pb levels in those papers, Pb levels by dry weight in the current study are shown in Table 1.

Materials and Methods

Samples and Pb analysis were same with chapter 3. As for the preparation of Pb analysis, samples of 100–300 mg of soft tissues were dried for 24 hours at 50°C. Our preliminary experiment showed that 24 hours were enough for drying the samples.

Table 1. Sample information, assessment of P exposure and hepatic Pb levels in both wet and dry weight.

Year	Species	Places	Sex	Age	Pb level (mg/kg) wet wt.	Pb level (mg/kg) dry wt.	Assessments
2016	Steller's sea eagle	Hokkaido	unk.	unk.	11.51	39.88	Pb poisoning
2016	Steller's sea eagle	Hokkaido	F	unk.	0.08	0.24	Non toxic
2016	Steller's sea eagle	Hokkaido	unk.	unk.	14.80	53.49	Pb poisoning
2016	Steller's sea eagle	Hokkaido	unk.	ad.	17.67		Pb poisoning
2016	Steller's sea eagle	Hokkaido	unk.	unk.	0.10		Non toxic
2016	Steller's sea eagle	Hokkaido	F	ad.	7.94	22.16	Pb poisoning
2016	Steller's sea eagle	Hokkaido	F	ad.	0.10	0.37	Non toxic
2016	Steller's sea eagle	Hokkaido	unk.	unk.	0.05	0.12	Non toxic
2016	Steller's sea eagle	Hokkaido	unk.	unk.	0.07	0.20	Non toxic
2017	Steller's sea eagle	Hokkaido	M	ad.	0.07	0.17	Non toxic
2017	Steller's sea eagle	Hokkaido	F	ad.	0.07	0.27	Non toxic
2017	Steller's sea eagle	Hokkaido	unk.	unk.	20.45	71.14	Pb poisoning
2017	Steller's sea eagle	Hokkaido	unk.	unk.	0.11	0.32	Non toxic
2017	Steller's sea eagle	Hokkaido	M	ad.	0.11	0.31	Non toxic
2017	Steller's sea eagle	Hokkaido	F	ad.	0.57	2.07	High Pb exposure
2017	Steller's sea eagle	Hokkaido	M	ad.	35.36	141.05	Pb poisoning
2017	Steller's sea eagle	Hokkaido	F	sub.	0.80	2.42	High Pb exposure
2017	Steller's sea eagle	Hokkaido	F	ad.	0.07	0.11	Non toxic
2017	Steller's sea eagle	Hokkaido	F	sub.	0.05	0.16	Non toxic
2017	Steller's sea eagle	Hokkaido	unk.	unk.	0.05	0.13	Non toxic
2017	Steller's sea eagle	Hokkaido	M	ad.	0.05	0.17	Non toxic
2015	White-tailed sea eagle	Hokkaido	unk.	unk.	50.79	126.26	Pb poisoning
2015	White-tailed sea eagle	Hokkaido	unk.	unk.	0.09	0.29	Non toxic

2015	White-tailed sea eagle	Hokkaido	F	sub.	0.15	0.46	Non toxic
2015	White-tailed sea eagle	Hokkaido	F	juv.	0.17	0.56	Non toxic
2016	White-tailed sea eagle	Hokkaido	unk.	unk.	0.07	0.18	Non toxic
2016	White-tailed sea eagle	Hokkaido	F	ad.	0.12	0.47	Non toxic
2016	White-tailed sea eagle	Hokkaido	F	juv.	0.15	0.49	Non toxic
2016	White-tailed sea eagle	Hokkaido	F	juv.	0.12	0.33	Non toxic
2016	White-tailed sea eagle	Hokkaido	F	juv.	0.07	0.21	Non toxic
2016	White-tailed sea eagle	Hokkaido	unk.	unk.	0.16	0.42	Non toxic
2016	White-tailed sea eagle	Hokkaido	unk.	unk.	0.03	0.08	Non toxic
2016	White-tailed sea eagle	Hokkaido	F	ad.	0.44	1.19	High Pb exposure
2017	White-tailed sea eagle	Hokkaido	unk.	unk.	17.54	55.76	Pb poisoning
2017	White-tailed sea eagle	Hokkaido	F	sub.	0.07	0.21	Non toxic
2017	White-tailed sea eagle	Hokkaido	♂	sub.	0.08	0.20	Non toxic
2017	White-tailed sea eagle	Hokkaido	unk.	unk.	0.16	0.56	Non toxic
2017	White-tailed sea eagle	Hokkaido	♂	sub.	0.10	0.31	Non toxic
2017	White-tailed sea eagle	Hokkaido	unk.	sub.	0.06	0.21	Non toxic
2017	White-tailed sea eagle	Hokkaido	unk.	unk.	0.16	0.57	Non toxic
2017	White-tailed sea eagle	Hokkaido	unk.	unk.	0.05	0.18	Non toxic
2017	White-tailed sea eagle	Hokkaido	unk.	unk.	0.03	0.10	Non toxic
2017	White-tailed sea eagle	Hokkaido	F	ad.	26.29	90.53	Pb poisoning
2017	White-tailed sea eagle	Hokkaido	M	sub.	0.03	0.11	Non toxic
2017	White-tailed sea eagle	Hokkaido	M	ad.	0.09	0.28	Non toxic
2017	White-tailed sea eagle	Hokkaido	M	ad.	0.07	0.21	Non toxic
2017	White-tailed sea eagle	Hokkaido	F	ad.	0.13	0.42	Non toxic
2017	White-tailed sea eagle	Hokkaido	M	ad.	0.08	0.24	Non toxic
2016	Mountain hawk-eagle	Hokkaido	unk.	unk.	0.70		High Pb exposure
2017	Mountain hawk-eagle	Hokkaido	unk.	unk.	0.10	0.31	Non toxic
2015	Mountain hawk-eagle	Honshu	unk.	unk.	0.05	0.16	Non toxic
2017	Mountain hawk-eagle	Hokkaido	M	sub.	0.12	0.45	Non toxic

2015	Blakiston's fish-owl	Hokkaido	F	unk.	0.04	0.13	Non toxic
2015	Blakiston's fish-owl	Hokkaido	F	unk.	0.06	0.14	Non toxic
2016	Blakiston's fish-owl	Hokkaido	M	sub.	0.02	0.07	Non toxic
2017	Blakiston's fish-owl	Hokkaido	unk.	ch.	0.06	0.24	Non toxic
2017	Blakiston's fish-owl	Hokkaido	F	juv.	0.07	0.20	Non toxic
2016	Blakiston's fish-owl	Hokkaido	M	sub.	0.02	0.08	Non toxic
2016	Peregrine falcon	Hokkaido	F	juv.	0.09	0.35	Non toxic
2015	Peregrine falcon	Honshu	unk.	unk.	0.09	0.30	Non toxic
2015	Eurasian Hobby	Hokkaido	unk.	unk.	0.28	0.84	High Pb exposure
2016	Eurasian Hobby	Hokkaido	M	juv.	0.09	0.38	Non toxic
2015	Northern goshawk	Hokkaido	F	unk.	0.14	0.53	Non toxic
2016	Northern goshawk	Honshu	unk.	unk.	0.16	0.51	Non toxic
2016	Sparrowhawk	Hokkaido	F	sub.	0.06	0.20	Non toxic
2015	Jungle crow	Hokkaido	M	unk.	0.06	0.21	Non toxic
2015	Jungle crow	Hokkaido	M	unk.	0.11	0.34	Non toxic
2016	Ural owl	Hokkaido	F	ad.	0.14	0.42	Non toxic
2016	Whooper swan	Honshu	unk.	unk.	0.18	0.58	Non toxic
2016	Whooper swan	Honshu	unk.	unk.	19.58	62.98	Pb poisoning
2016	Whooper swan	Hokkaido	M	unk.	19.56	69.70	Pb poisoning
2016	Greater white-fronted goose	Honshu	unk.	unk.	0.10	0.31	Non toxic
2016	Greater white-fronted goose	Honshu	unk.	unk.	0.72	2.57	High Pb exposure

Note 3-2

Pb distribution in organs of Pb-exposed birds

Background and Objective

Hepatic concentrations of Pb in carcasses or blood levels of Pb in live birds are generally used to assess Pb exposure in birds. This is because concentrations of Pb are generally highest in the blood immediately after absorption, with a short half-life of hours, followed by the liver and kidneys, with a half-life ranging from days to months. However, there are anatomical differences among birds, and the ability to metabolize Pb can be different depending on the species. The objective in this study was to clarify the Pb distribution in soft tissues in birds.

Materials and Methods

Sampling

The carcasses of Steller's sea eagles (*Haliaeetus pelagicus*, $n = 3$) and whooper swans (*Cygnus cygnus*, $n = 2$) that likely died due to Pb poisoning or were exposed to abnormal levels of Pb, according to clinical signs and hepatic Pb levels, were used. Sample information is shown in Table 1. Specimens were provided by the Institute for Raptor Biomedicine Japan. Specimens of liver, kidney, blood, bile, spleen, ovary, oviduct, testis, cerebrum, cerebellum, thyroid gland, adrenal gland, lung, intestine, stomach contents, pancreas, and muscle were collected, although collected specimens were limited because of other tests or research.

Pb analysis

An analysis of Pb followed the protocol described in chapter 3.

Results and Discussion

In this very limited number of samples, Pb was widely distributed in the organs (Table 1). In eagles, hepatic Pb concentration was the highest, whereas the renal Pb concentration was the highest (but very similar to that in the liver) in swans (Table 1 and 2). Pb was also accumulated in the brain, which confirmed that Pb can pass through the blood-brain barrier. Pb-poisoned birds show signs of neurotoxicity, and therefore the brain has a high risk of damage due to Pb exposure. Bile, spleen, and genitals showed high accumulations of Pb, and the Pb level in muscle was low compared to that in other tissues. Therefore, Pb causes adverse effects in many organs.

Table 1. Sample information.

The locations of the sampling are shown in Figure S1.

Species	Year	Sex	Location
Steller's sea eagle_1	2017	m	Hokkaido
Steller's sea eagle_2	2015	f	Hokkaido
Steller's sea eagle_3	2014	f	Hokkaido
Whooper swan_1	2016	m	Hokkaido
Whooper swan_2	Unknown	m	Miyagi

m: male; f: female

Table 2. Pb concentrations in organs（mg/kg, wet weight）

Species	Liver	Kidney	Blood	Bile	Spleen	Ovary	Oviduct	Testis
Steller's sea eagle_1	39.4	29.2	7.8	17.7	6.1			1.6
Steller's sea eagle_2	32.9	8.5		29.8		4.6		
Steller's sea eagle_3	18.3	4.6			0.56			
Whooper swan_1	19.6	21.3		4.8	7.5			1.9
Whooper swan_2	19.6	26.4		3.1				0.75

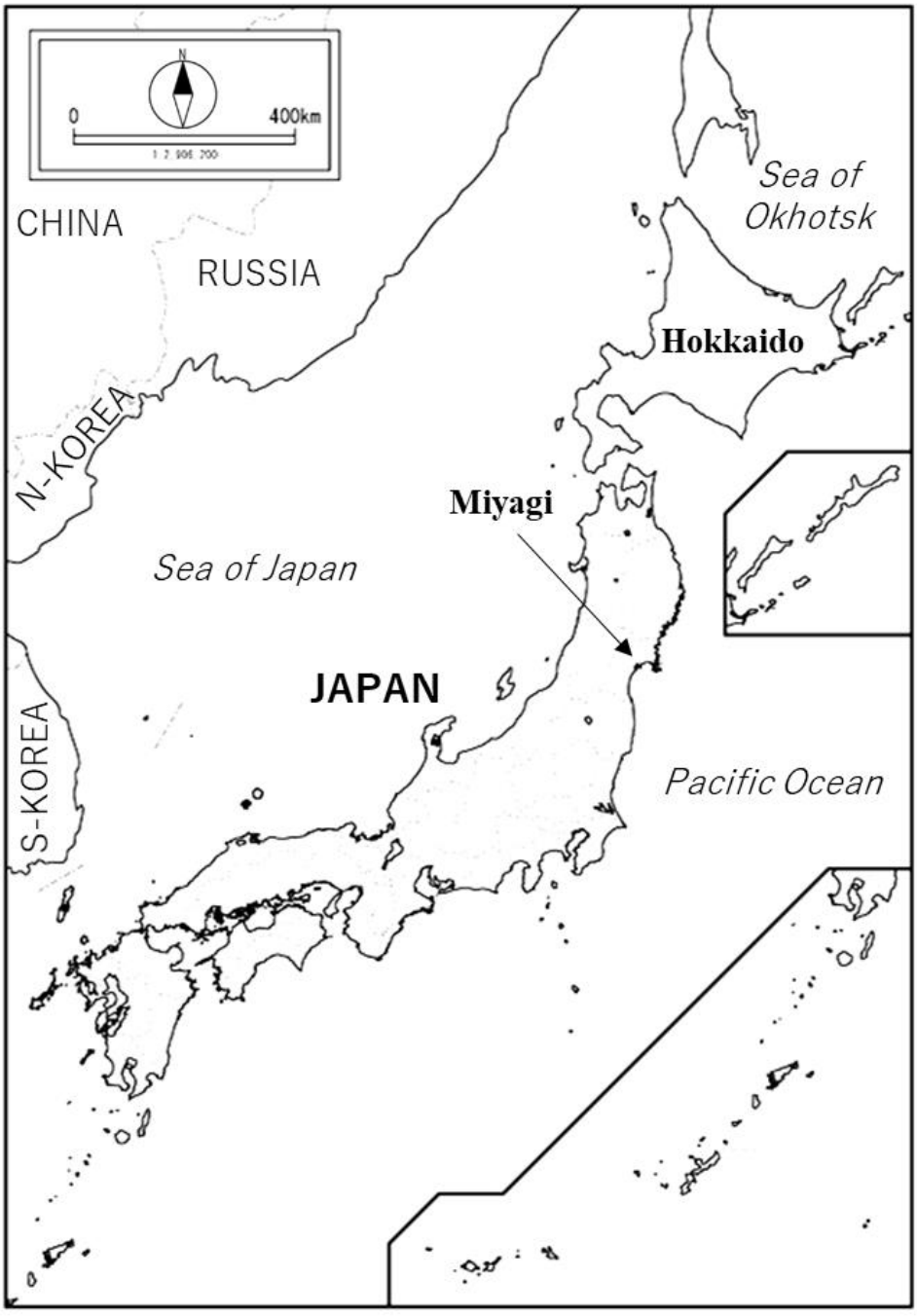
Species	Cerebrum	Cerebellum	Thyroid gland	Adrenal gland	Lung	Intestine	Stomach contents	Pancreas	Muscle
Steller's sea eagle_1			1.2	2.0	3.1	7.6	1.9 (glandular) / 23.0 (gizzard)		0.35
Steller's sea eagle_2									0.44
Steller's sea eagle_3	1.1	2.0	0.50	0.29	1.1		0.58		0.11
Whooper swan_1			2.8	1.5					
Whooper swan_2									0.69

Table 3. The ratio of Pb concentrations between liver and other organs

Species	Liver	Kidney	Blood	Bile	Spleen	Ovary	Oviduct	Testis
Steller's sea eagle_1	1	0.7	0.2	0.4	0.2			0.04
Steller's sea eagle_2	1	0.3		0.9	0.0	0.1		
Steller's sea eagle_3	1	0.3						
Whooper swan_1	1	1.1		0.2	0.4			0.1
Whooper swan_2	1	1.3		0.2				

Species	Cerebrum	Cerebellum	Thyroid gland	Adrenal gland	Lung	Intestine	Stomach contents	Pancreas	Muscle
Steller's sea eagle_1			0.03	0.1	0.1	0.2	0.05		0.01
Steller's sea eagle_2									0.01
Steller's sea eagle_3	0.1	0.1	0.03	0.02	0.1		0.03		0.01
Whooper swan_1			0.1	0.1					
Whooper swan_2									0.04

Figure S1. The locations of the sampling.



CHAPTER 4

Lead distribution in bones of lead-poisoned eagles and swans; bone samples as useful indicators

Abstract

Lead (Pb) poisoning in raptors and waterbirds is a serious problem in many countries. However, only a fraction of Pb poisoning has been identified in birds. Bone specimens may be useful indices of Pb exposure because bones contain ~90% of the total Pb body burden. The purpose of this study was to first comprehensively analyze Pb accumulation in bone types from the entire body using inductively coupled plasma-mass spectrometry (ICP-MS). These results showed that Pb accumulation differed greatly depending on bone type, and trabecular bone and bones that contain bone marrow accumulated high levels of Pb. Therefore, a second purpose was to investigate the detailed Pb distribution and the relation with bone structure or bone marrow by imaging elements using laser ablation (LA)-ICP-MS. This study determined several routes of Pb accumulation in the bones of avian species. Our findings suggested that bone specimens that (1) mainly consist of trabecular bones and (2) contain bone marrow accumulate high levels of Pb. The shorter turnover time of trabecular bone can cause a rapid accumulation of Pb, and bone marrow may have an important role in carrying Pb into bones. Pb is accumulated in bones via blood flow, and bone marrow receives blood from outside the bones. In conclusion, bone specimens have valuable information on Pb exposure and would be useful to investigate and understand mortalities related to suspected Pb poisoning.

Key words

Lead (Pb) poisoning, Eagle, Swan, Bone, Imaging

Highlights

- Bone specimens have differing Pb accumulation depending on bone type, and that bones can be effectively used to understand Pb poisoning in birds.
- Bone specimens that (1) mainly consist of trabecular bones and (2) contain bone marrow accumulate high levels of Pb.
- Reconsideration of toxic levels in humerus or femur for the assessment of Pb exposure is needed

1. Introduction

Many raptors and waterbirds worldwide die due to lead (Pb) poisoning in many countries (Berny et al., 2015; Fisher et al., 2006; Kendall et al., 1996; Scheuhammer and Norris, 1996). Pb is introduced to their environment through Pb ammunition, which shatter into fragments on impact, or fishing equipment. Raptors ingest Pb from prey that have been shot with Pb ammunition (Saito, 2009), and waterbirds ingest Pb fragments from shot pellets or fishing sinkers when they take small stones for gastroliths (Martinez-Haro et al., 2011)(Capter 2 and 3).

Pb exposure may cause a variety of sub-lethal toxic effects, reduced survivability, and direct mortality (Kendall et al., 1996). Sub-lethal effects of Pb are exerted on the nervous system, kidneys and circulatory system, resulting in physiological, biological and behavioral changes (Fisher et al., 2006; Scheuhammer, 1987). As a result of these changes, birds may become increasingly susceptible to predation, starvation and infection of various diseases, increasing the probability of death from other causes (Fisher et al., 2006; Scheuhammer and Norris, 1996).

Because many birds around the world are dead due to Pb exposure, it is important to understand Pb poisoning for the conservation of birds. Species with international statuses as endangered, vulnerable or threatened species have reportedly ingested or been poisoned by Pb ammunition fragments. Pb poisoning has been reported in the Californian condor (*Gymnogyps californianus*), white-rumped vulture (*Gyps bengalensis*), Spanish imperial eagle (*Aquila adalberti*), whooping crane (*Grus americana*), Steller's sea eagle (*Haliaeetus pelagicus*) and white-tailed sea eagle (*Haliaeetus albicilla*). It is of key importance to conservation that Pb-related mortality can be avoided for these species (Fisher et al., 2006).

In Hokkaido, the northern island of Japan, many endangered Steller's sea eagles and white-tailed sea eagles are dying due to Pb poisoning despite regulations minimizing the introduction of Pb to their environment (Saito, 2009). Many waterbirds, such as swans, are also exposed to Pb in Japan (Honda et al., 1990) and various countries (Haig et al., 2014; Langner et al., 2015; Madry et al., 2015). However, only a fraction of Pb poisoning has been identified. Therefore, a greater understanding of Pb poisoning in birds is necessary to improve the regulation and conservation of avian species.

Concentrations of Pb are generally highest in the blood immediately after absorption, with a short half-life of hours, followed by the liver and kidneys, with a half-life ranging from days to months. Bones have the longest Pb half-life, and Pb deposited in bone can remain for years (Fisher et al., 2006; Pain, 1996). Although liver or blood specimens are traditionally used as indices of Pb exposure, it might be difficult to obtain these soft tissues from the natural environment because the internal organs of carcasses are frequently consumed by other animals. Bone specimens, on the other hand, remain in the environment, and many bones are kept in museums or universities, and might be useful for analysis.

Bones contain 84%–90% of the total Pb body burden in raptors(García-Fernández et al., 1997), and Pb can substitute for Ca in hydroxyapatite of bones (Ellis et al., 2006). Previous studies investigating Pb exposure in birds using bones have focused on the humerus or femur (Mateo et al., 2003; Pain et al., 2005). The accumulation patterns of Pb differ depending on the structure and function of the bone type. Therefore, an understanding of Pb distribution in bones throughout the entire body would be useful for accurately monitoring Pb poisoning in birds.

Laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) plays a key role as a microanalytical technique. It enables multi-element trace analysis at the trace and ultra-trace level, and has been used to produce images of detailed, regionally-specific element distribution in thin sections of tissues (Becker et al., 2007; Limbeck et al., 2015). Pb distribution, as well as other metals, in human teeth has been analyzed using LA-ICP-MS (Hare et al., 2011).

The purpose of this study was to first investigate Pb accumulation in various bone types from the entire body of birds using ICP-MS. Bone types that could potentially be used as indicators for Pb exposure were discussed. The second purpose was to analyze more detailed accumulations of Pb in one bone type, by imaging of elements using LA-ICP-MS to reveal a route of Pb accumulation. To our knowledge, this is the first study to perform LA-ICP-MS analysis in avian bones to investigate Pb exposure. Detailed imaging of Pb distribution and the comparison of Pb localization with other metals in bones should help us understand the accumulation mechanisms of Pb, and accurately assess Pb exposure in birds.

2. Materials and Methods

2.1. Sampling

The carcasses of Steller's sea eagle (n=3), white-tailed sea eagle (n=2), whooper swan (*Cygnus cygnus*, n=2) and swan (species is unknown) (n=2) that have likely died due to Pb poisoning, according to clinical signs and hepatic Pb levels were collected. A mountain hawk-eagle (*Spizaetus nipalensis*, n=1) that died due to other causes in a Pb free environment was used as a control. The Steller's sea eagles, one of the white-tailed sea eagles, one of the swans and the mountain hawk-eagle were provided by the Kushiro Nature Conservation Office, Ministry of the Environment, and Institute for Raptor Biomedicine Japan. Another white-tailed sea eagle was provided by the Hokkaido Institute of Public Health. Three of the swans were provided by the Ibaraki Prefectural Government, Environmental Policy Division. Bones (cranial bone, hyoid bone, atlas, axis, 3rd and 4th cervical vertebrae, notarium, scapula, sternum, keel, coracoideum, costa vertebralis, costa sternalis, ilium, pygostyle, humerus, radius, ulna, phalanx distalis digiti majoris, femur, patella, tibiotarsus, fibula, tarsometatarsus, and os digiti pedis), spinal cord specimens, and bone marrow (axis, radius, ulna, tibiotarsus, and tarsometatarsus) were collected from the birds (Fig. S1). Bone marrow from the femur was collected in all swans. For the vertebrae, the spinous process was collected. For the long bones, diaphysis was selected. For the femur and tibiotarsus, diaphysis (proximal, medial, and distal) and epiphysis (surface and core in proximal and distal) were collected (Fig. S2). Two specimens were collected from same parts of all bone types and the average concentration of the two specimens was used. The femur and tibiotarsus were specifically selected for this study to understand the differences between swans and eagles based on the presence or absence of bone marrow. Femur bones in swans contain bone marrow, whereas femur

bones in eagles do not. Both birds contain bone marrow in the tibiotarsus. For ease of duplication in measurement, approximately two specimens were collected from one region of bone. The average concentration from each region was used for the analysis. Several bone parts could not be collected for the analysis, because they had been used for taxidermy or had already been removed. Bone specimens were kept at -20°C.

2.2. Analysis of Pb concentration using ICP-MS

The analysis of Pb concentration followed the method in Yabe et al (Yabe et al., 2015). Samples were briefly dried and digested with nitric acid (Kanto Chemical Corporation, Tokyo, Japan) and hydrogen peroxide (Kanto Chemical Corporation) in a microwave digestion system (Speedwave Two, Berghof, Germany). Pb concentration was measured with an inductively coupled plasma-mass spectrometer (ICP-MS; 7700 series, Agilent Technology, Tokyo, Japan). More details regarding the pretreatment and mass spectrometry are presented in Text S1 and Table S1.

2.3. Analysis of Pb distribution using LA-ICP-MS

The bones were briefly washed and embedded in epoxy resin, sliced to ~40 μm sections along the transversal axis, and polished. The bone sections were systematically scanned by a focused laser beam using LA (NWR213; ESI, Portland, OR, USA)-ICP-QQQ (triple quadrupole)-MS (8800 series; Agilent Technologies, Tokyo, Japan). We reconstructed images of LA-ICP-MS by iQuant2 (Suzuki et al., submitted to Journal of Mass Spectrometry Society of Japan). More details regarding the pretreatment and mass spectrometry are presented in Text S2 and Table S2.

2.4. Statistics

Pearson product-moment correlation (r) was used to analyze the relationships among the bones, livers, and kidneys of eagles and swans, with a significance level of $p < 0.05$. Statistical analyses were performed in JMP Pro 12 (SAS Institute, Cary, NC).

3. Results

3.1. Differences in Pb concentration depend on the bone type

There were large differences in Pb concentration depending on the bone type. In eagles, the axis, hyoid (around the greater horn), keel, pygostyle and patella had higher Pb concentrations (Fig 1). In swans, the hyoid bone, scapula, keel, costa vertebralis and pygostyle had higher Pb concentrations (Fig 2). Although the Pb concentration in one swan (D) was lower than in the other three swans, it exhibited similar Pb distribution patterns (Fig. S3). The levels of Pb in bone marrow were not high compared to trabecular bones. The mountain hawk-eagle that was used as a control had low Pb concentrations (less than 0.7 mg/kg) in all bones.

Steller's sea eagle (C) had higher Pb concentrations (dry weight) in the axis (127.9 mg/kg), hyoid (110.2 mg/kg), and keel (28.9 mg/kg) than the humerus (diaphysis, 3.6 mg/kg) and femur (diaphysis, 4.9 mg/kg). Hepatic Pb concentration in the eagle was very high (58.4 mg/kg, dry weight), indicating serious Pb poisoning.

Within the femur and the tibiotarsus, Pb concentrations in the epiphysis (which mainly consisted of trabecular bone) were substantially higher than the diaphysis (which mainly consisted of cortical bone) for both eagles and swans (Fig 3-a and -b). Furthermore, in the tibiotarsus, the proximal epiphysis that included bone marrow had the highest concentration. In swans, femur that included bone marrow showed higher Pb concentration than humerus that was devoid of bone marrow. In eagles, the femur and humerus both lacked bone marrow and showed similar Pb levels. The level of Pb in bone marrow itself sometimes differed depending on whether it was obtained from the proximal or distal part of the same bone (the average is shown in Fig 1 and 2, and the data of each level is not shown).

There were significant correlations in Pb concentrations between various types of bones and the liver or kidney in both eagles and swans (Table S3 and Fig. S4). The correlations between the humerus and the kidney or liver were especially high (humerus-kidney: $r = 1.00$ and humerus-liver: $r = 0.88$, $p < 0.05$, respectively).

3.2. Detailed distribution pattern of Pb in one bone using LA-ICP-MS

Photomicrographs of the femur and tibiotarsus of Steller's sea eagle are shown in Fig S5, and Pb distribution in the femur and tibiotarsus of Steller's sea eagle and whooper swan by imaging is shown in Fig 4. The epiphysis in all four bone types had higher signal intensity count per second (cps) of Pb than the diaphysis. The inner parts of the diaphysis of the bones showed high Pb cps. In the epiphysis of femur from whooper swan or tibiotarsus from Steller's sea eagle and whooper swan, the surface of the trabecular bone had high cps. However, the femur of adult Steller's sea eagle differed from the other bones; Pb accumulation in the surface of the hollow epiphysis was slightly high, but the cps did not differ greatly.

When compared to the accumulation of other metals, areas which accumulated high cps of Pb showed low cps of Ca (Fig 5). Fe distribution, which indicates the presence of bone marrow or blood, had a different distribution pattern to Pb (Fig. S6). Local distributions of ^{13}C, ^{25}Mg, ^{31}P, ^{55}Mn, ^{57}Fe, ^{65}Cu, ^{66}Zn, ^{206}Pb and ^{207}Pb in the femur and tibiotarsus of Steller's sea eagle and whooper swan are shown in Fig. S7-S10.

4. Discussion

4.1. Importance of trabecular bone and bone marrow in Pb accumulation

Our findings strongly suggest that trabecular bone and bones with active marrow can play an important role in understanding Pb toxicity, as they tend to readily accumulate Pb in bones of avian species.

The hyoid, keel and pygostyle bones that mainly consist of the trabecular bone type had remarkably high Pb concentration in both eagles and swans (Fig 1 and 2). In the femur and tibiotarsus, the epiphysis had a higher Pb level than the diaphysis (Fig 3-a and -b). These levels of Pb concentration differ because the diaphysis is mainly composed of cortical bone, whereas the epiphysis is mainly composed of trabecular bone. This trend is supported by a previous study, which found that, in humans, Pb in trabecular bones is more biologically active than Pb in cortical bones (Barbosa Jr et al., 2005). The authors of that study also reported that trabecular bone had a larger surface area and received a greater volume of blood compared to cortical bone. Moreover, the shorter turnover time of trabecular bone causes a rapid accumulation of Pb. The half-life of Pb in trabecular bone may vary from a few years in vertebrae to 16 years, whereas the half-life in cortical bone often ranges from 5-15 years, but may exceed 25 years (Gerhardsson et al., 2005). Additionally, other trabecular bone, such as the axis, scapula, costa and patella, were found to have higher concentrations of Pb than cortical bone, such as the diaphysis of the humerus, radius, ulna and tarsometatarsus (Fig 1 and 2).

Bones containing bone marrow are another factor determining Pb accumulation, because Pb in bone marrow is carried into bones through blood vessels. Avian bone marrow consists of cell rich and highly vascularized tissues (Higgins, 1999; Tavassoli and Yoffey, 1983), which may result in significant Pb exposure. The Pb level in bone marrow

was not particularly high, which may be attributed to the shorter half-life of Pb in blood (about 1 month (Hu et al., 1998)). The Pb level in the femur was higher in swans than that in eagles. Eagle femurs do not contain bone marrow, whereas swan femurs do. In the tibiotarsus, the proximal epiphysis that contained bone marrow had the highest concentration of Pb. The peripheral portion of the marrow cylinder is hyperplastic in adult birds, whereas the axial portion is hypoplastic and contains a large amount of fat (Higgins, 1999; Jordan and Robeson, 1942). These characteristics may explain the differences in Pb concentrations in bone marrow from the same bones.

Age related differences in Pb accumulation may also exist, because young and adult birds have different bone structure and functions of bone marrow. During postnatal development, a large portion of the skeleton becomes pneumatized, displacing hemopoietic bone marrow (Schepelmann, 1990). In this study, age differences were not evaluated as a factor because the sample number was limited (Steller's sea eagle (A): unknown; Steller's sea eagle (B): adults; Steller's sea eagle (C): adult; white-tailed sea eagle (A): sub-adult; white-tailed sea eagle (B): adult; all swans: adult). Samples of young birds were difficult to obtain as some of the species are classified as endangered species. Further research may clarify the relationship between Pb distribution in bones and differences due to age.

4.2. Necessity of reconsidering the assessment of Pb exposure

In raptors, Pb concentrations >10 mg/kg in the humerus or femur indicates abnormal exposure (Mateo et al., 2003). In waterbirds, Pb concentration of 20 mg/kg in the femur indicates a high level of Pb exposure (Pain et al., 2005).

This study showed that, although Pb levels in the bones of Steller's sea eagle (C) and swan (C) were lower than the reference levels, the concentrations in the liver and kidney were remarkably high (Fig 1 and 2). The humerus had a significantly high correlation with the liver (r = 0.88) and kidney (r = 1.00), suggesting that this bone may be a good indicator of Pb exposure. However, according to the comparison with hepatic Pb level, the toxic levels of Pb in the humerus or femur should be lower than the reference levels. Therefore, reconsideration of toxic levels in humerus or femur for the assessment is needed. On the other hand, trabecular bones, such as the hyoid, keel or pygostyle, could be potential indicators for Pb poisoning in birds because they tend to accumulate Pb faster and at higher levels than other bones.

In avian species, acute Pb poisoning and mortality can follow the ingestion of a single Pb gunshot (Fisher et al., 2006; Pain and Rattner, 1988). Pb levels in the cortical bone are a better dosimeter of long-term cumulative Pb exposure than in trabecular bone because of the long half-life of Pb in humans (Hu et al., 1998). In rats and humans, trabecular bone is reported to be a better indicator for acute or intermediate Pb poisoning (Brito et al., 2014; Gerhardsson et al., 1993). Acute Pb exposure in birds is different from rats or humans because birds may accidently ingest Pb directly through scavenging prey shot with Pb ammunition or mispurposing bullets for gastroliths. Furthermore, there is a possibility that wild birds are chronically exposed to sub-lethal levels of Pb (Kendall et al., 1996). The Pb distribution patterns in bone would be different depending on the circumstances surrounding Pb exposure. In cases of acute poisoning, Pb level in trabecular bone could be significantly higher than in cortical bone, whereas in cases of chronic exposure, the differences of concentration between trabecular and cortical bone may be insignificant.

4.3. Detailed Pb distribution patterns in femur and tibiotarsus bones using imaging

The comparison of Pb concentrations in various bone types using ICP-MS indicated that trabecular bone and bones that contain bone marrow accumulate acute amounts of Pb levels. Therefore, further and more detailed analyses of Pb distribution using LA-ICP-MS were performed to verify this finding, and similar trend was confirmed.

The comparison of Ca and Pb distributions also indicated that Pb accumulation may be faster in trabecular bones than in cortical bones. The regions with high Pb cps showed low Ca cps (Fig 5), suggesting that the replacement of Ca with Pb, or Pb accumulation, may have started from bone parts with lower density. In humans, the bone density of trabecular bone is lower than the bone density of cortical bone (Lang et al., 2004). From the ICP-MS results, Ca concentrations in bones were significantly higher (hundreds to thousands of times) than Pb concentrations. Therefore, it is difficult to identify the replacement of Ca from LA-ICP-MS imaging. The analysis of the eagles that were not exposed to Pb would show the normal distribution of Ca. The comparison between Pb exposed bones and healthy bones may indicate the interaction between Ca replacement and Pb accumulation. A previous study on vultures suggested that the mineralization degree of bones decreases as Pb accumulation increases (Gangoso et al., 2009). This current study showed that Pb and Ca may interact during Pb poisoning in birds.

Bones containing bone marrow are an important driver of the differences in Pb distribution in bones. The main difference between the femur of Steller's sea eagle and other three bone parts (the tibiotarsus of Steller's sea eagle and whooper swan, and the femur of whooper swan) was the presence or absence of bone marrow. The femur of the

adult Steller's sea eagle did not contain bone marrow, whereas the three other bone types did. The detailed analysis of the diaphysis and epiphysis in these other bones showed that bone parts in contact with bone marrow had high Pb accumulations. Meanwhile, the femur of adult Steller's sea eagle had slightly high Pb accumulation in the surface of the hollow epiphysis, but the level did not differ greatly to the inside of it. Therefore, bone marrow may have an important role in carrying Pb into bones. Pb is accumulated in bones via blood flow, and bone marrow receives blood from outside the bones.

Pb concentrations in bone marrow, and comparisons of Pb and Fe distributions, showed that bone marrow itself did not have higher Pb cps than trabecular bone (Fig 1, 2 and S6). The half-life of Pb in blood is around one month (Hu et al., 1998), which is significantly shorter than bones, resulting in lower Pb cps in bone marrow.

5. Conclusions

Our findings suggest that bone specimens have differing Pb accumulation depending on bone type, and that bones can be effectively used to understand Pb poisoning in birds. In Japan, many raptors and waterbirds die due to Pb poisoning, despite regulations against indiscriminately introducing Pb into the environment. Further research on Pb accumulation in bones will allow bones to be used as an indicator for Pb toxicity levels in avian species. This would greatly increase our knowledge of Pb poisoning in birds and allow us to develop more appropriate regulations.

Fig. 1. Pb distribution in bone and organ tissues specimens from Steller's sea eagle (n=3) and white-tailed sea eagle (n=2). The position of each bone is shown in S1 Fig. with the skeleton diagram of each bird.

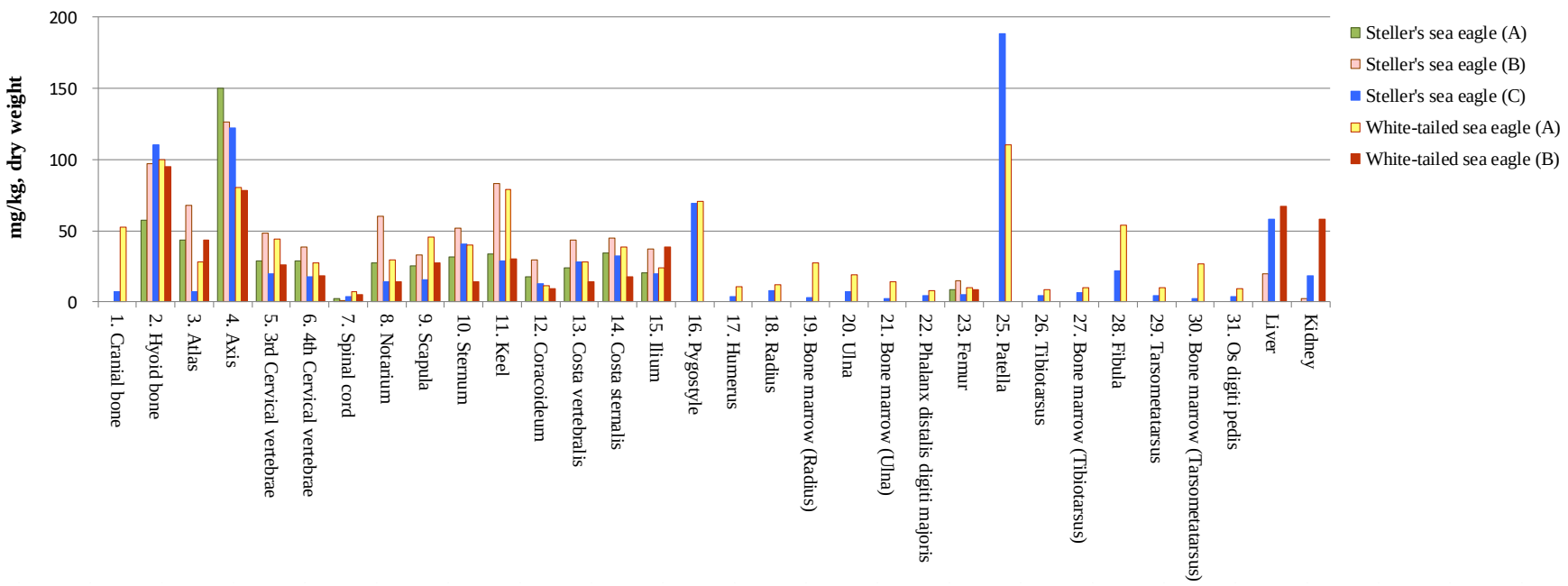


Fig. 2. Pb distribution in bone and organ tissues from swans (n=4). The data of swan (D) was shown in S3 Fig; an expanded version of this figure. The position of each bone is shown in S1 Fig.

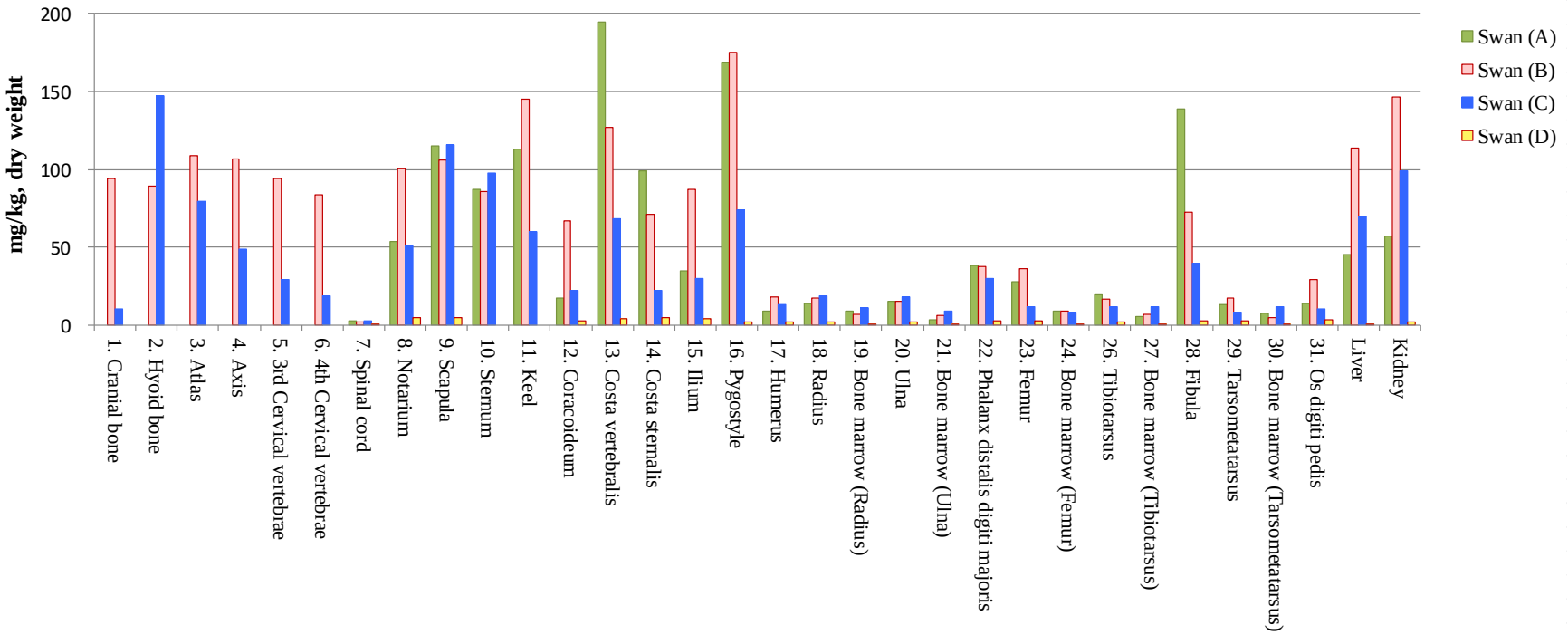
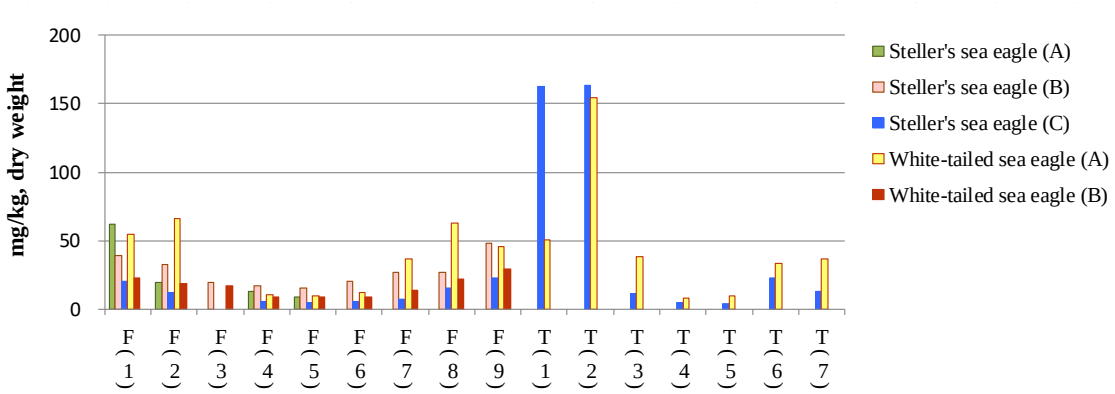


Fig. 3. Pb concentrations in the diaphysis and the epiphysis in femur (F) and tibiotarsus (T) in eagles (a) and swans (b). (1): epiphysis (proximal, surface); (2): epiphysis (proximal, core); (3): diaphysis (proximal, internal struts); (4): diaphysis (proximal); (5): diaphysis (medial); (6): diaphysis (distal); (7): diaphysis (distal, internal struts); (8): epiphysis (distal, core); and (9): epiphysis (distal, surface). The position of each part is shown in Fig. S2.

a)



b)

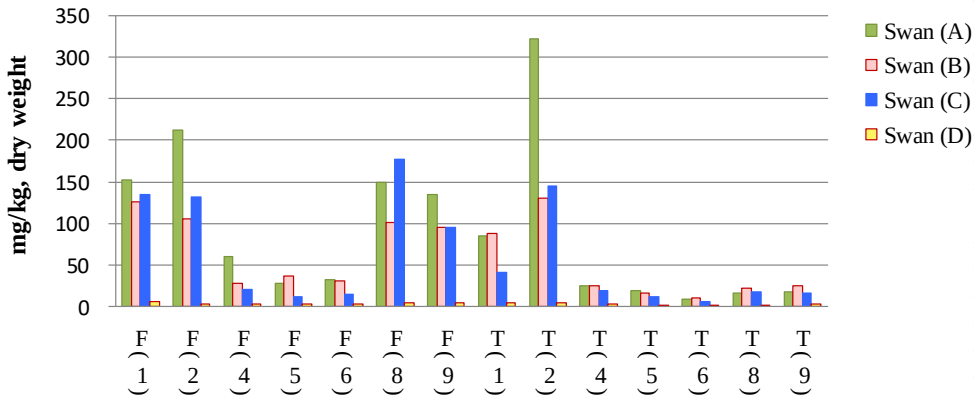


Fig. 4. Pb distribution in femur (distal) and tibiotarsus (proximal) of Steller's sea eagle and whooper swan using LA-ICP-MS.

1) femur of Steller's sea eagle, 2) tibiotarsu of Steller's sea eagle, 3) femur of whooper swan, 4) tibiotarsus of whooper swan. The figures on the bottom row are an expanded version of a, b and c in 1), and 2). The ranges of count per second (cps) of Pb differ depending on the images.

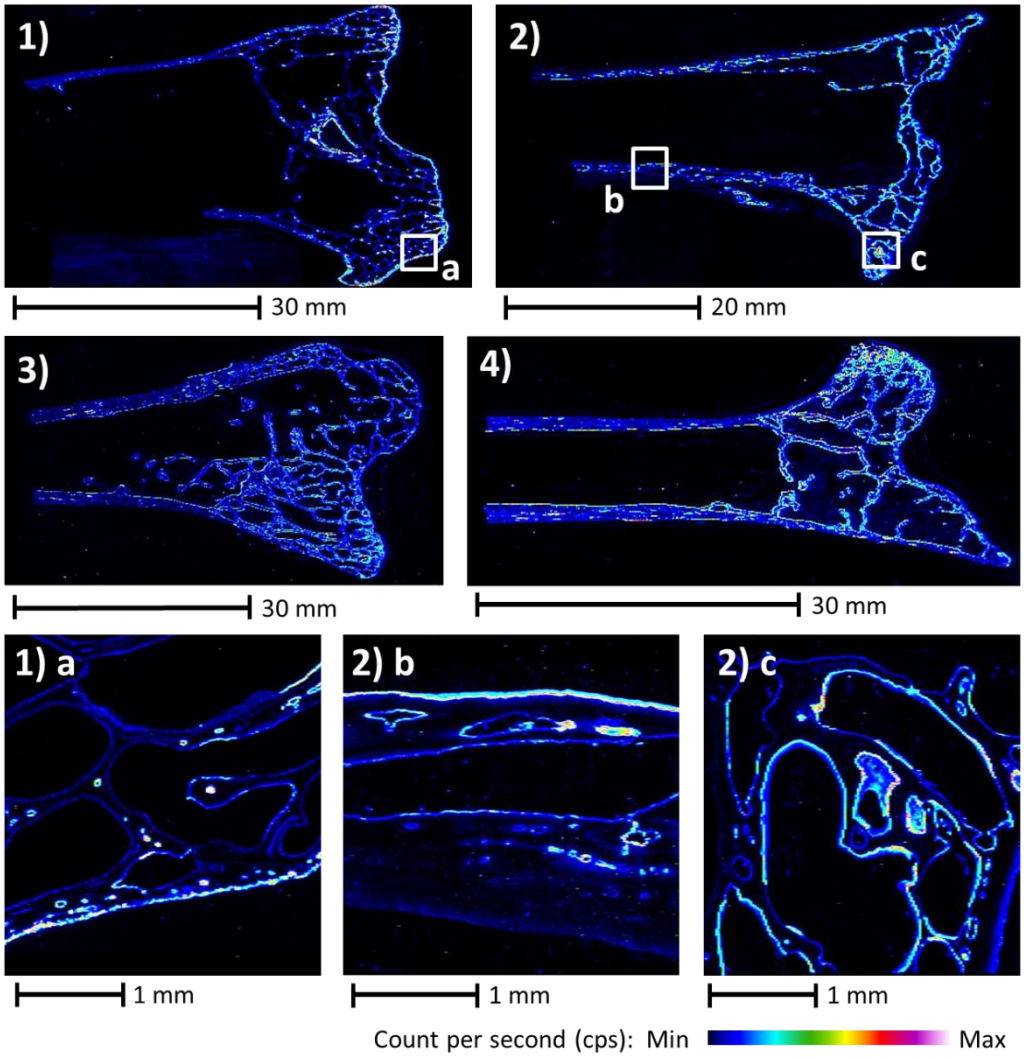
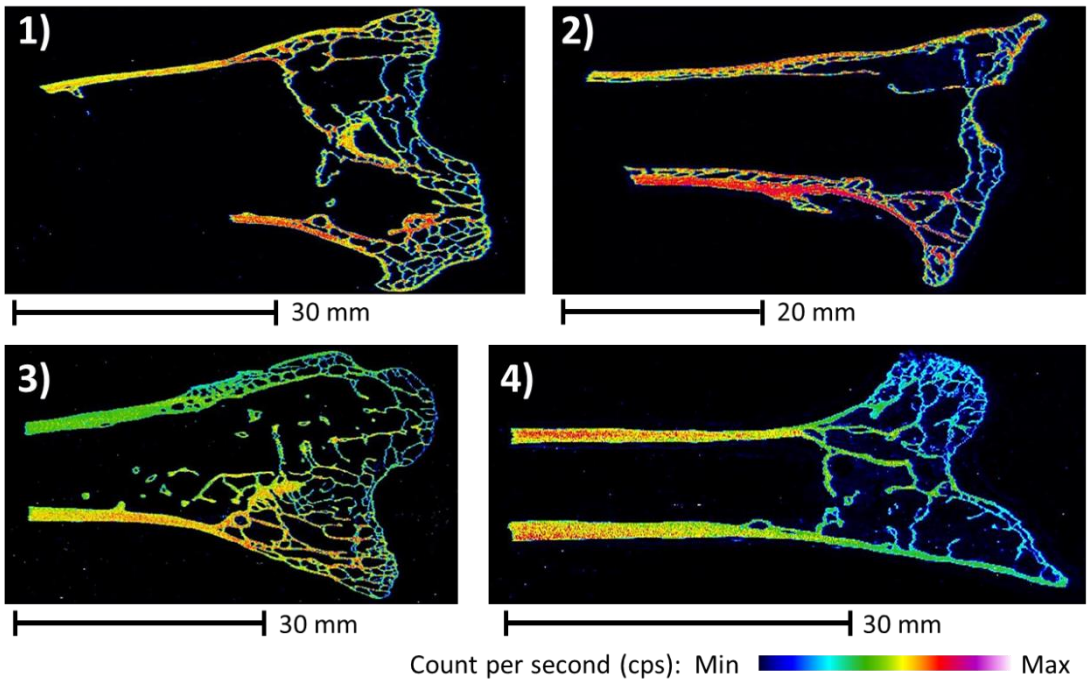


Fig. 5. Ca distribution in femur (distal) and tibiotarsus (proximal) of Steller's sea eagle and whooper swan using LA-ICP-MS.

1) Femur of Steller's sea eagle, 2) tibiotarsu of Steller's sea eagle, 3) femur of whooper swan, 4) tibiotarsus of whooper swan. The ranges of count per second (cps) of Ca differ depending on the images.



References

Barbosa Jr, F., Tanus-Santos, J.E., Gerlach, R.F., Parsons, P.J., 2005. A critical review of biomarkers used for monitoring human exposure to lead: advantages, limitations, and future needs. Environ. Health Perspect. 1669–1674.

Becker, J.S., Zoriy, M., Becker, J.S., Dobrowolska, J., Matusch, A., 2007. Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) in elemental imaging of biological tissues and in proteomics. J. Anal. At. Spectrom. 22, 736–744.

Berny, P., Vilagines, L., Cugnasse, J.-M., Mastain, O., Chollet, J.-Y., Joncour, G., Razin, M., 2015. VIGILANCE POISON: Illegal poisoning and lead intoxication are the main factors affecting avian scavenger survival in the Pyrenees (France). Ecotoxicol. Environ. Saf. 118, 71–82.

Brito, J.A.A., Costa, I.M., e Silva, A.M., Marques, J.M.S., Zagalo, C.M., Cavaleiro, I.I.B., Fernandes, T.A.P., Gonçalves, L.L., 2014. Changes in bone Pb accumulation: Cause and effect of altered bone turnover. Bone 64, 228–234.

Ellis, D.E., Terra, J., Warschkow, O., Jiang, M., González, G.B., Okasinski, J.S., Bedzyk, M.J., Rossi, A.M., Eon, J.-G., 2006. A theoretical and experimental study of lead substitution in calcium hydroxyapatite. Phys. Chem. Chem. Phys. 8, 967–976.

Fisher, I.J., Pain, D.J., Thomas, V.G., 2006. A review of lead poisoning from ammunition sources in terrestrial birds. Biol. Conserv. 131, 421–432. doi:http://dx.doi.org/10.1016/j.biocon.2006.02.018

Gangoso, L., Álvarez-Lloret, P., Rodríguez-Navarro, A.A.B., Mateo, R., Hiraldo, F., Donázar, J.A., 2009. Long-term effects of lead poisoning on bone mineralization in vultures exposed to ammunition sources. Environ. Pollut. 157, 569–574.

García-Fernández, A.J., Motas-Guzmán, M., Navas, I., Maria-Mojica, P., Luna, A., Sánchez-García, J.A., 1997. Environmental exposure and distribution of lead in four species of raptors in southeastern Spain. Arch. Environ. Contam. Toxicol. 33, 76–82.

Gerhardsson, L., Akantis, A., Lundström, N.-G., Nordberg, G.F., Schütz, A., Skerfving, S., 2005. Lead concentrations in cortical and trabecular bones in deceased smelter workers. J. Trace Elem. Med. Biol. 19, 209–215.

Gerhardsson, L., Attewell, R., Chettle, D.R., Englyst, V., Lundström, N.G., Nordberg, G.F., Nyhlin, H., Scott, M.C., Todd, A.C., 1993. In vivo measurements of lead in bone in long-term exposed lead smelter workers. Arch. Environ. Heal. An Int. J. 48, 147–156.

Haig, S.M., D'Elia, J., Eagles-Smith, C., Fair, J.M., Gervais, J., Herring, G., Rivers, J.W., Schulz, J.H., 2014. The persistent problem of lead poisoning in birds from ammunition and fishing tackle. Condor 116, 408–428. doi:10.1650/CONDOR-14-36.1

Hare, D., Austin, C., Doble, P., Arora, M., 2011. Elemental bio-imaging of trace elements in teeth using laser ablation-inductively coupled plasma-mass spectrometry. J. Dent. 39, 397–403.

Higgins, J., 1999. Túnel: a case study of avian zooarchaeology and taphonomy. J. Archaeol. Sci. 26, 1449–1457.

Honda, K., Lee, D.P., Tatsukawa, R., 1990. Lead poisoning in swans in Japan. Environ. Pollut. 65, 209–218.

Hu, H., Rabinowitz, M., Smith, D., 1998. Bone lead as a biological marker in epidemiologic studies of chronic toxicity: conceptual paradigms. Environ. Health Perspect. 106, 1-8.

Jordan, H.E., Robeson, J.M., 1942. The production of lymphoid nodules in the bone marrow of the domestic pigeon, following splenectomy. Am. J. Anat. 71, 181–205.

Kendall, R.J., Lacker, T.E., Bunck, C., Daniel, B., Driver, C., Grue, C.E., Leighton, F., Stansley, W., Watanabe, P.G., Whitworth, M., 1996. An ecological risk assessment of lead shot exposure in non-waterfowl avian species: Upland game birds and raptors. Environ. Toxicol. Chem. 15, 4–20. doi:10.1002/etc.5620150103

Lang, T., LeBlanc, A., Evans, H., Lu, Y., Genant, H., Yu, A., 2004. Cortical and trabecular bone mineral loss from the spine and hip in long-duration spaceflight. J. bone Miner. Res. 19, 1006–1012.

Langner, H.W., Domenech, R., Slabe, V.A., Sullivan, S.P., 2015. Lead and Mercury in Fall Migrant Golden Eagles from Western North America. Arch. Environ. Contam. Toxicol. 1–8.

Limbeck, A., Galler, P., Bonta, M., Bauer, G., Nischkauer, W., Vanhaecke, F., 2015. Recent advances in quantitative LA-ICP-MS analysis: challenges and solutions in the life sciences and environmental chemistry. Anal. Bioanal. Chem. 407, 6593–6617.

Madry, M.M., Kraemer, T., Kupper, J., Naegeli, H., Jenny, H., Jenni, L., Jenny, D., 2015. Excessive lead burden among golden eagles in the Swiss Alps. Environ. Res. Lett. 10, 034003.

Martinez-Haro, M., Taggart, M.A., Martín-Doimeadiós, R.R.C., Green, A.J., Mateo, R., 2011. Identifying Sources of Pb Exposure in Waterbirds and Effects on Porphyrin Metabolism Using Noninvasive Fecal Sampling. Environ. Sci. Technol. 45, 6153–6159. doi:10.1021/es2009242

Mateo, R., Taggart, M., Meharg, A.A., 2003. Lead and arsenic in bones of birds of prey from Spain. Environ. Pollut. 126, 107–114.

Pain, D.J., 1996. Lead in waterfowl. Lewis Publishers: New York.

Pain, D.J., Meharg, A.A., Ferrer, M., Taggart, M., Penteriani, V., 2005. Lead concentrations in bones and feathers of the globally threatened Spanish imperial eagle. Biol. Conserv. 121, 603–610.

Pain, D.J., Rattner, B.A., 1988. Mortality and hematology associated with the ingestion of one number four lead shot in black ducks, *Anas rubripes*. Bull. Environ. Contam. Toxicol. 40, 159–164.

Saito, K., 2009. Lead poisoning of Steller's Sea-Eagle (*Haliaeetus pelagicus*) and Whitetailed Eagle (*Haliaeetus albicilla*) caused by the ingestion of lead bullets and slugs. Hokkaido Japan. RT Watson, M. Fuller, M. Pokras, WG Hunt (Eds.). Ingestion Lead from Spent Ammunit. Implic. Wildl. Humans. Peregrine Fund, Boise, Idaho, USA.

Schepelmann, K., 1990. Erythropoietic bone marrow in the pigeon: development of its distribution and volume during growth and pneumatization of bones. J. Morphol. 203, 21–34.

Scheuhammer, A.M., 1987. The chronic toxicity of aluminium, cadmium, mercury, and lead in birds: A review. Environ. Pollut. 46, 263–295. doi:http://dx.doi.org/10.1016/0269-7491(87)90173-4

Scheuhammer, A.M., Norris, S.L., 1996. The ecotoxicology of lead shot and lead fishing weights. Ecotoxicology 5, 279–295.

Tavassoli, M., Yoffey, J.M., 1983. Bone marrow, structure and function. AR Liss.

Yabe, J., Nakayama, S.M.M., Ikenaka, Y., Yohannes, Y.B., Bortey-Sam, N., Oroszlany, B., Muzandu, K., Choongo, K., Kabalo, A.N., Ntapisha, J., Mweene, A., Umemura, T., Ishizuka, M., 2015. Lead poisoning in children from townships in the vicinity of a lead-zinc mine in Kabwe, Zambia. Chemosphere 119, 941–947. doi:10.1016/j.chemosphere.2014.09.028

Supplementary data

Text S1. Analysis of Pb concentrations using ICP-MS.

Text S2. Analysis of Pb distributions using LA-ICP-MS.

Table S1. Detailed analytical conditions of ICP-MS.

Table S2. Detailed analytical conditions of LA-ICP-MS.

Table S3. Pairwise correlation coefficients (*r*) for Pb levels between bones and livers or kidneys.

Fig. S1. The position of bone and bone marrow specimens collected from birds.

Fig. S2. The position of bone and bone marrow specimens collected from birds.

Fig. S3. Pb distribution in bone and organ tissues in swan (D).

Fig. S4. Correlation coefficients between bones and liver or kidney.

Fig. S5. Photomicrographs of femur (1) and tibiotarsus (2) of Steller's sea eagle.

Fig. S6. Fe 1) and Pb 2) distribution in tibiotarsus (proximal) of whooper swan using LA-ICP-MS.

Fig. S7. Local distributions of ^{13}C, ^{25}Mg, ^{31}P, ^{55}Mn, ^{57}Fe, ^{65}Cu, ^{66}Zn, ^{206}Pb and ^{207}Pb in femur of Steller's sea eagle.

Fig. S8. Local distributions of ^{13}C, ^{25}Mg, ^{31}P, ^{55}Mn, ^{57}Fe, ^{65}Cu, ^{66}Zn, ^{206}Pb and ^{207}Pb in tibiotarsus of Steller's sea eagle.

Fig. S9. Local distributions of ^{13}C, ^{25}Mg, ^{31}P, ^{55}Mn, ^{57}Fe, ^{65}Cu, ^{66}Zn, ^{206}Pb and ^{207}Pb in femur of whooper swan.

Fig. S10. Local distributions of ^{13}C, ^{25}Mg, ^{31}P, ^{55}Mn, ^{57}Fe, ^{65}Cu, ^{66}Zn, ^{206}Pb and ^{207}Pb in tibiotarsus of whooper swan.

Text S1. Analysis of Pb concentrations using ICP-MS.

For the metal analysis, 10–30 mg of bones and 100–300 mg of livers or kidneys were dried for 24 hours at 50°C. Our preliminary experiment showed that 24 hours were sufficient to dry the samples. Bones were washed with distilled water (Maruyama Manufacturing, Tokyo, Japan) and an ultrasonic generator to remove other tissues before drying. All samples were then digested with 5 mL of 30% nitric acid (Kanto Chemical Corporation) and 1 mL of 30% hydrogen peroxide (Kanto Chemical Corporation) in a microwave digestion system (Speedwave Two). The volume was then brought to 10 mL with 2% nitric acid. Digestion was performed under the following conditions: 180°C for 15 min, 200°C for 20 min, and 100°C for 20 min. Pb concentration was measured with ICP-MS (7700 series, Agilent Technology). The instrument was calibrated using standard Pb solutions to establish standard curves before analysis. All chemicals and standard stock solutions were pure analytical reagent grade (Wako Pure Chemicals Industries). Water was distilled and deionized (Milli-Q, Merck Millipore, Billerica, Massachusetts). Analytical quality control was performed using Bone Ash (National Institute of Standards and Technology, Gaithersburg, MD), DOLT-4 (dogfish liver) and DORM-3 (fish protein) (National Research Council of Canada, Ottawa, Canada) certified reference materials. Replicate analyses of these reference materials showed good recoveries (80%–102%). The detection limit for Pb was 0.01 μg/kg. For the analysis of Pb concentration, Thallium (205Tl) was used as an internal standard.

Text S2. Analysis of Pb distributions using LA-ICP-MS.

Bones were washed with distilled water (Maruyama Manufacturing) and an ultrasonic generator to remove other tissues from outside and inside the diaphysis, and were embedded in epoxy resin. Samples were sliced into ~40 μm sections along the transversal axis with a diamond blade and polished. The bone sections were systematically scanned by a focused laser beam with the following parameters; spot diameter: 100 μm (20 μm in zoomed cases), scan speed: 500 μm/sec (20 μm/sec) using LA (NWR213; ESI)-ICP-QQQ-MS (8800 series; Agilent Technologies). Detailed analytical conditions are presented in Table S2. LA-ICP-MS operating conditions were optimized using NIST 610 glass (National Institute of Standards and Technology, Gaithersburg, MD, USA). We reconstructed two-dimensional images from time resolved analysis data of LA-ICP-MS by iQuant2 (Suzuki et al., submitted to Journal of Mass Spectrometry Society of Japan); a software developed in-house. This software shows the localization of metals. The ranges of the metal concentrations in images differ depending on the samples.

Table S1. Detailed analytical conditions of ICP-MS.

Parameter	Value
RF Power	1500 W
Argon gas pressure	600 kPa
Cell gas (Helium)	100 kPa
Peak pattern	1
Replicates	3
Sweeps/replicate	100
Stabilization time	30 s

Table S2. Detailed analytical conditions of LA-ICP-MS.

LA system (NWR213, ESI, Portland, OR, USA)	
Wavelength, nm	213
Pulse duration, ns	4
Fluence	2.7 J/cm^2
Repetition rate	10 Hz
Spot diameter	100 µm (zoomed version: 20 µm)
Scan speed	500 µm/ sec (zoomed version: 20 µm/sec)
Ablation mode	line scan
Carrier He gas flow rate	0.8 L/min
Make up Ar gas flow rate	0.8 L/min

ICP-QQQ-MS (8800 series, Agilent Technologies, Tokyo, Japan)	
RF power	1550 W
Plasma Ar gas flow rate	15 L/min
Auxiliary Ar gas	not used
Collision	not used
MS/MS	not used
Integration time	0.01 sec for ^{206}Pb, ^{207}Pb, ^{208}Pb, and 0.005 sec for other isotopes
Measured Isotopes	^{13}C, ^{25}Mg, ^{31}P, ^{43}Ca, ^{55}Mn, ^{57}Fe, ^{65}Cu, ^{66}Zn, ^{206}Pb, ^{207}Pb, ^{208}Pb

Table S3. Pairwise correlation coefficients (*r*) for Pb levels between bones and livers or kidneys.

		r	*p*-value
Liver	Ilium	0.82	0.025
Liver	Humerus	0.88	0.049
Kidney	Humerus	1.00	< 0.001
Kidney	Os digiti pedis	0.91	0.032
Kidney	Radius	0.89	0.041
Kidney	Ilium	0.81	0.028

Fig. S1. The position of bone and bone marrow specimens collected from birds. The map of the skeleton diagram of bird was modified from "Squelette_oiseau.JPG: BIODIDAC derivative work: mario modesto (Squelette_oiseau.JPG)". The bone marrow in the femur (*[1]) exists only in swans, and patella was collected only from the eagles (*[2]) in this study.

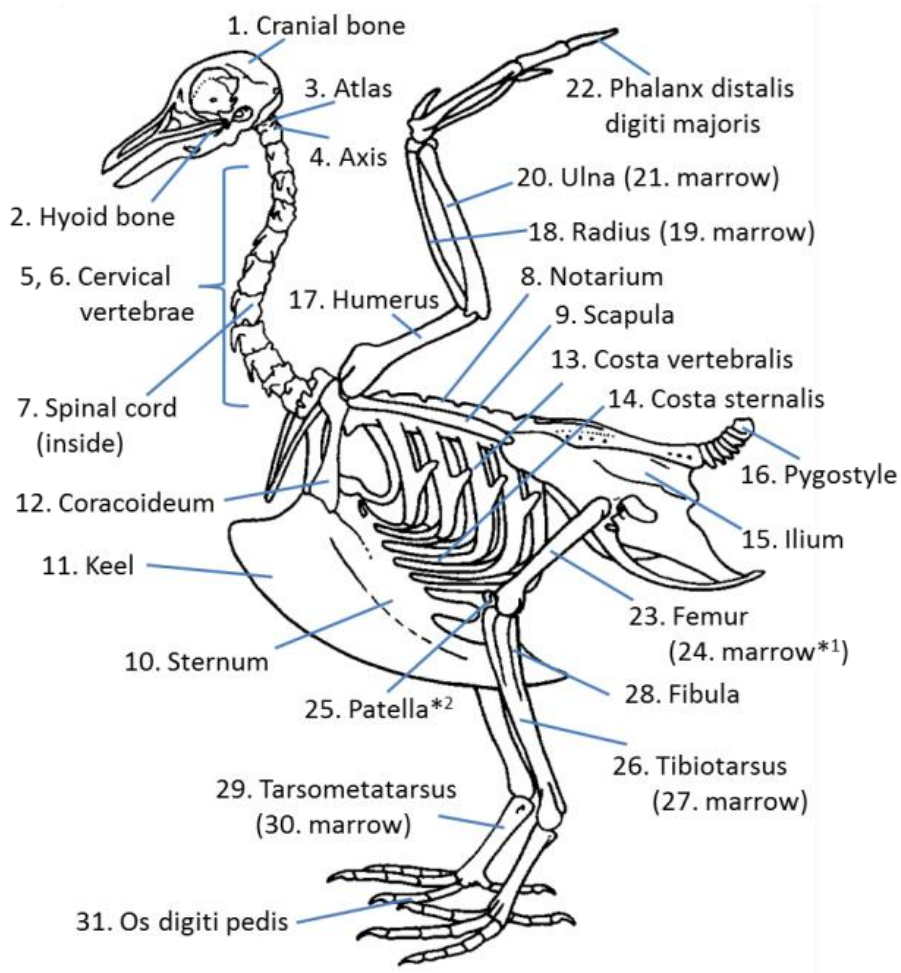


Fig. S2. The position of bone and bone marrow specimens collected from birds. The internal struts (3) were only collected from the femur in Steller's sea eagle (*[1]).

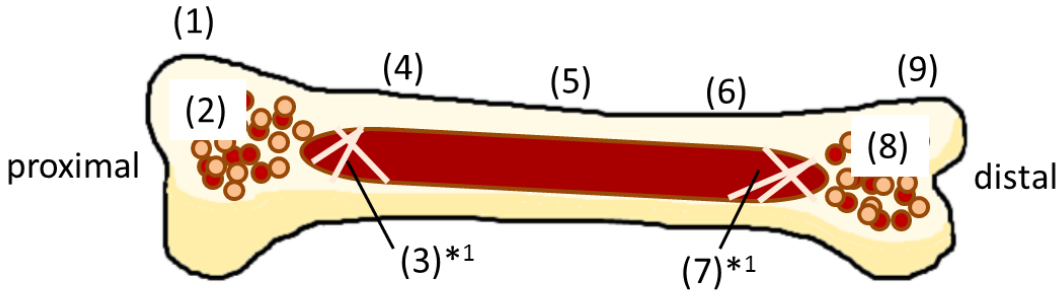


Fig. S3. Pb distribution in bone and organ tissues in swan (D). (The expanded version of Figure 2). Markers indicate the average of two specimens collected from the same bone.

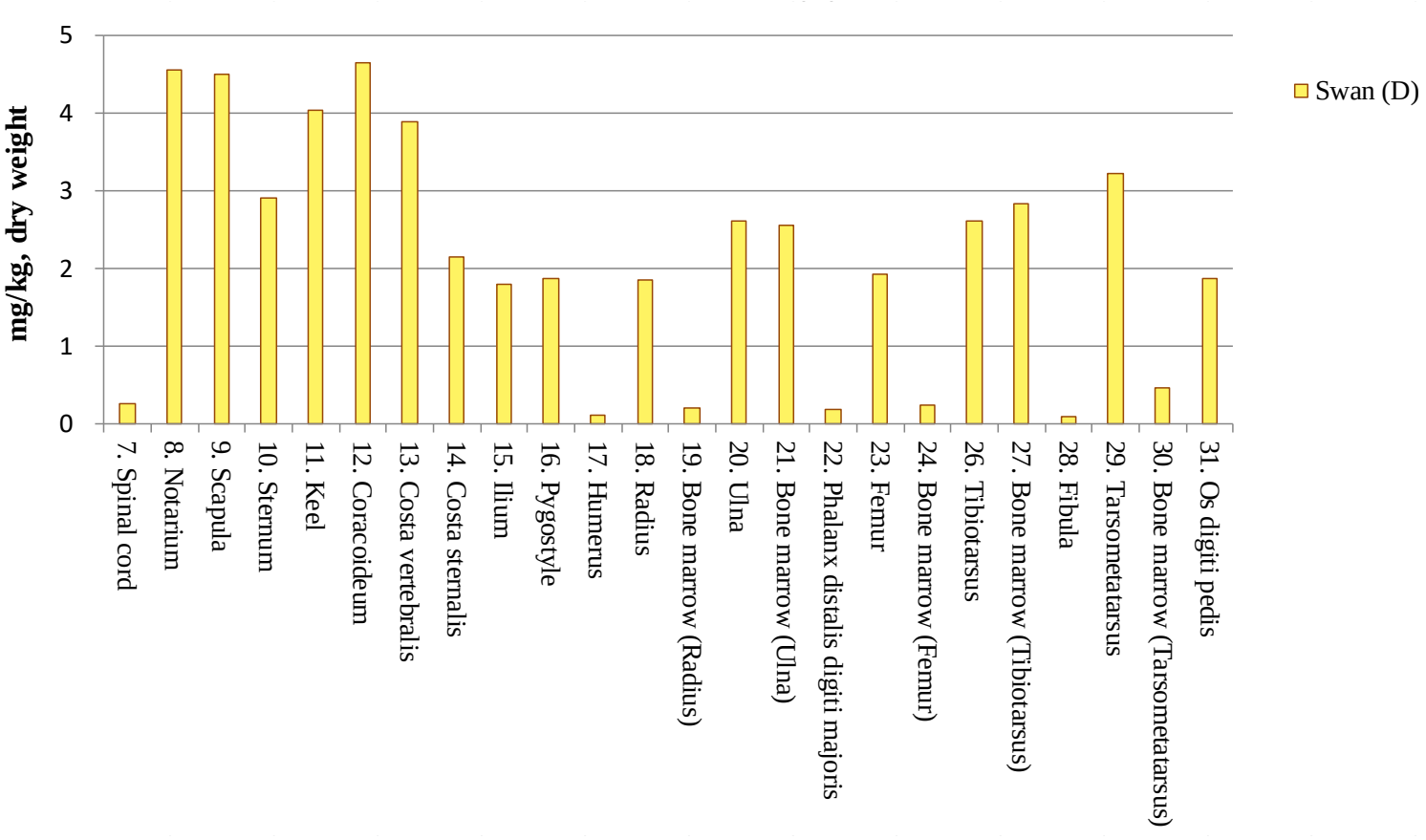


Fig. S4. Correlation coefficients between bones and liver or kidney.

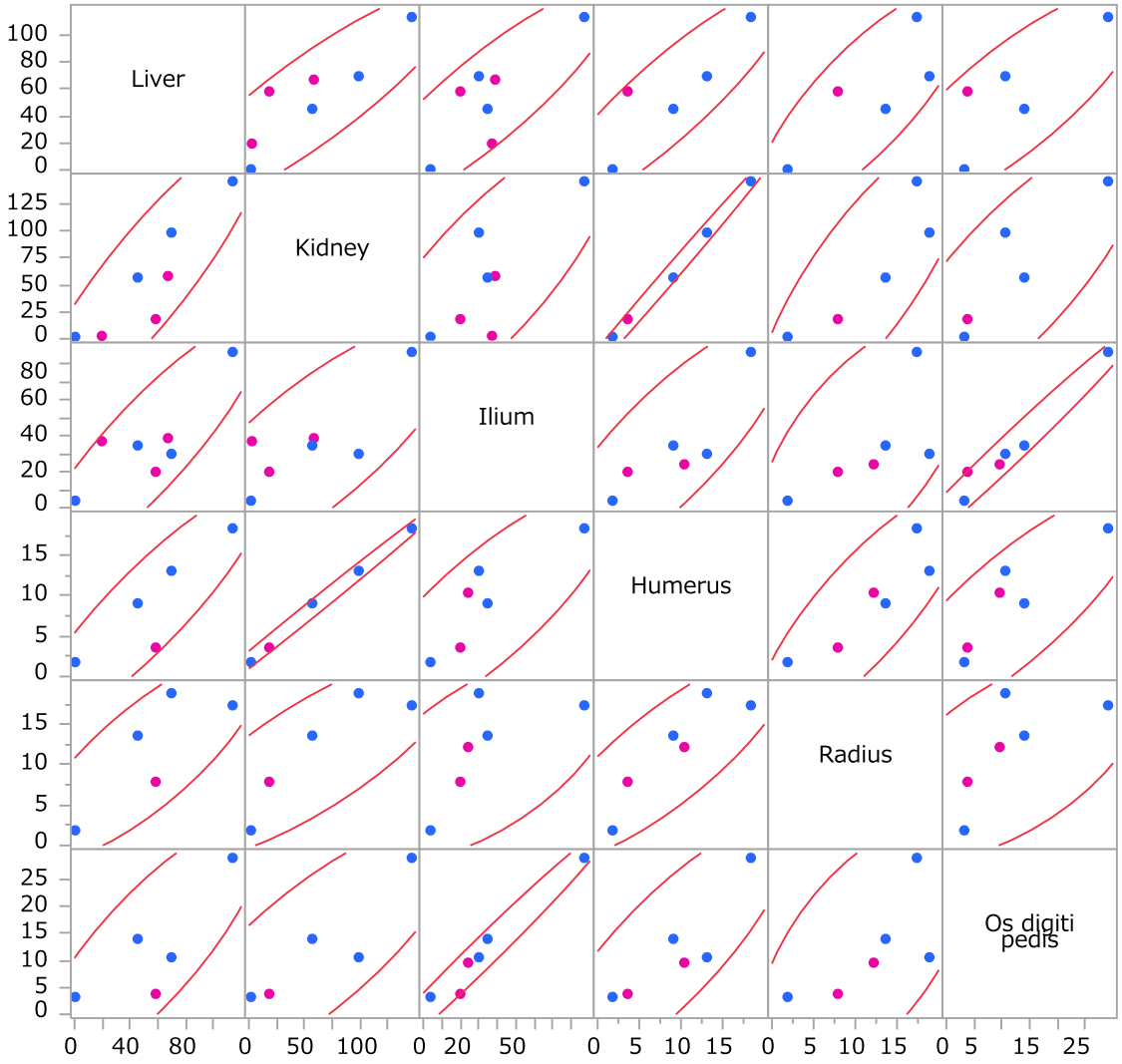
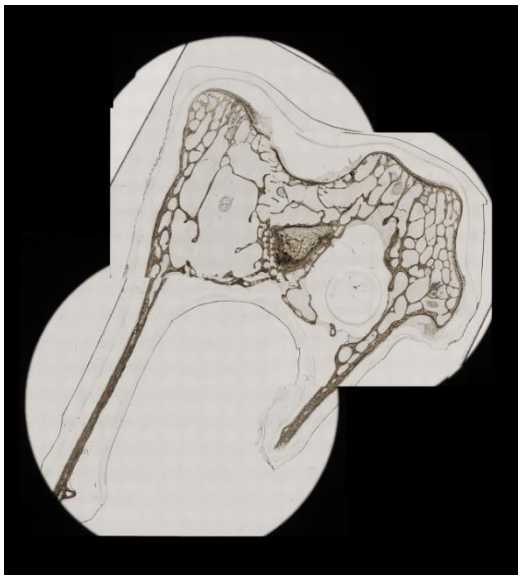


Fig. S5. Photomicrographs of femur (1) and tibiotarsus (2) of Steller's sea eagle. Due to the large size of the samples, three photomicrographs were combined to make one figure.

(1)



(2)

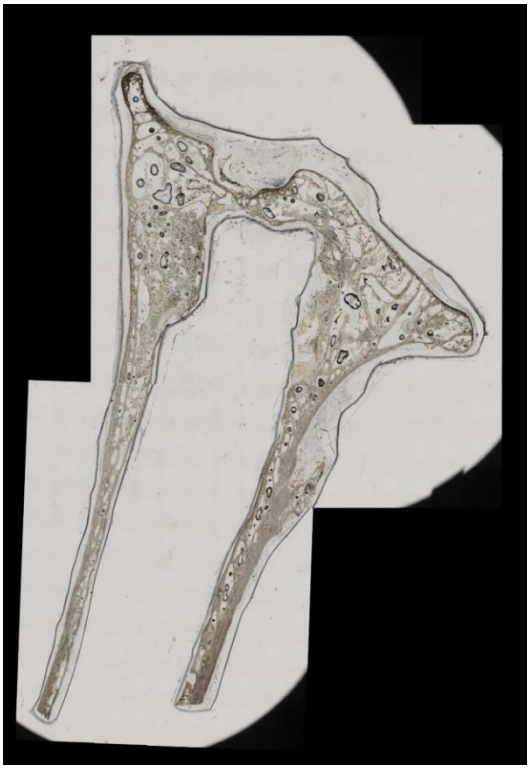


Fig. S6. Fe 1) and Pb 2) distribution in tibiotarsus (proximal) of whooper swan using LA-ICP-MS. The ranges of count per second (cps) of metals differ depending on the images.

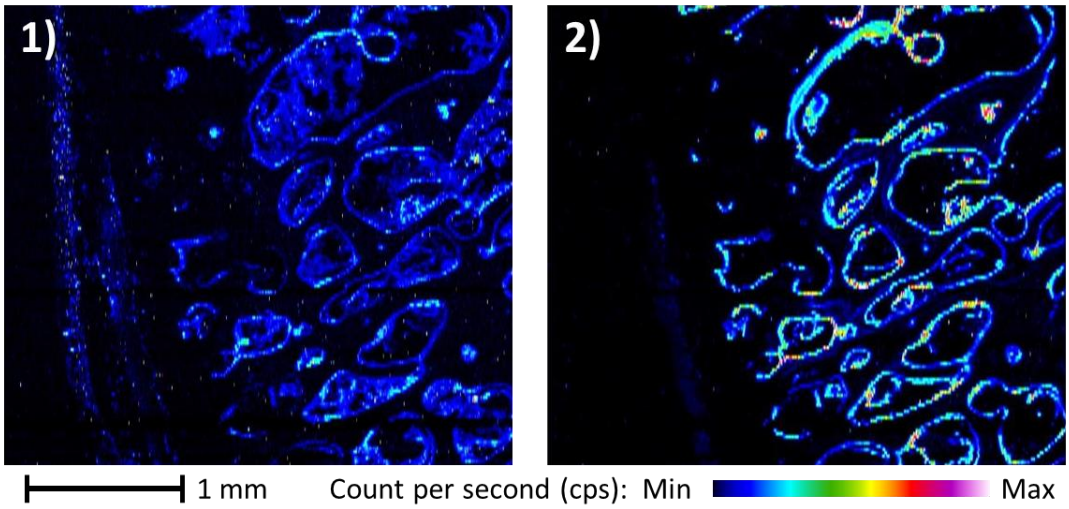


Fig. S7. Local distributions of ^{13}C, ^{25}Mg, ^{31}P, ^{55}Mn, ^{57}Fe, ^{65}Cu, ^{66}Zn, ^{206}Pb and ^{207}Pb in femur of Steller's sea eagle. The ranges of count per second (cps) of metals differ depending on the images.

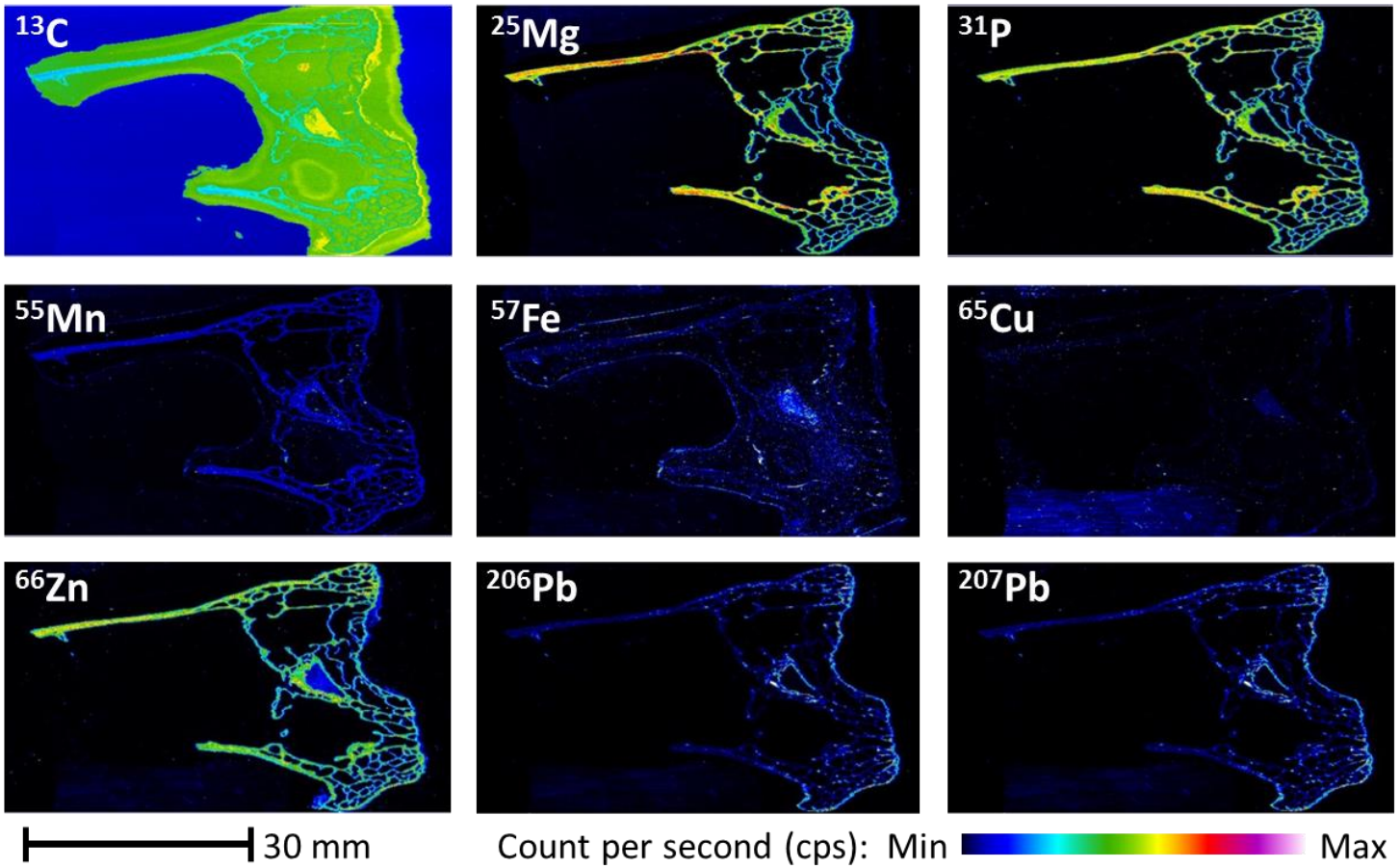


Fig. S8. Local distributions of ^{13}C, ^{25}Mg, ^{31}P, ^{55}Mn, ^{57}Fe, ^{65}Cu, ^{66}Zn, ^{206}Pb and ^{207}Pb in tibiotarsus of Steller's sea eagle. The ranges of count per second (cps) of metals are different depending on the images.

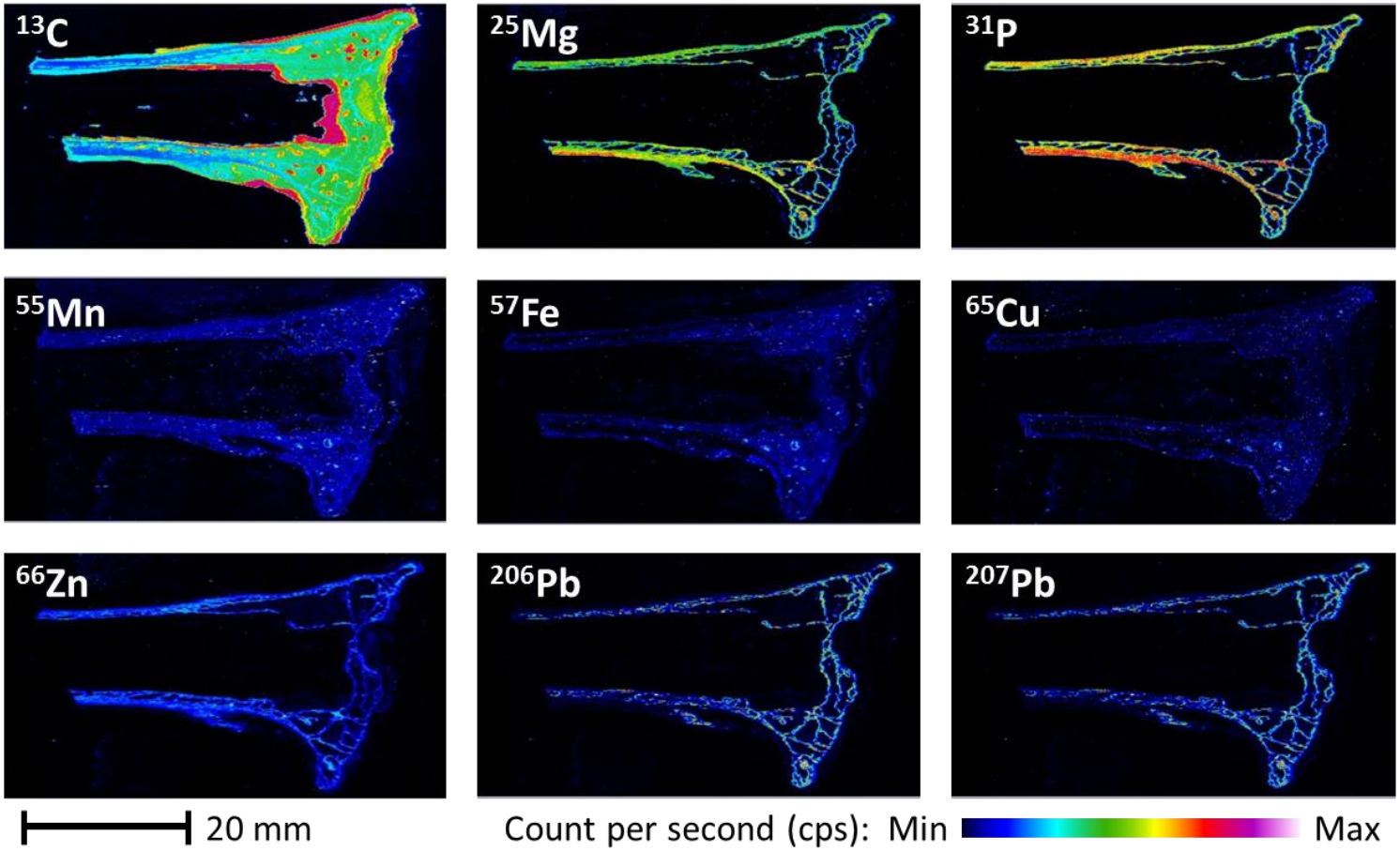


Fig. S9. Local distributions of ^{13}C, ^{25}Mg, ^{31}P, ^{55}Mn, ^{57}Fe, ^{65}Cu, ^{66}Zn, ^{206}Pb and ^{207}Pb in femur of whooper swan. The ranges of count per second (cps) of metals differ depending on the images.

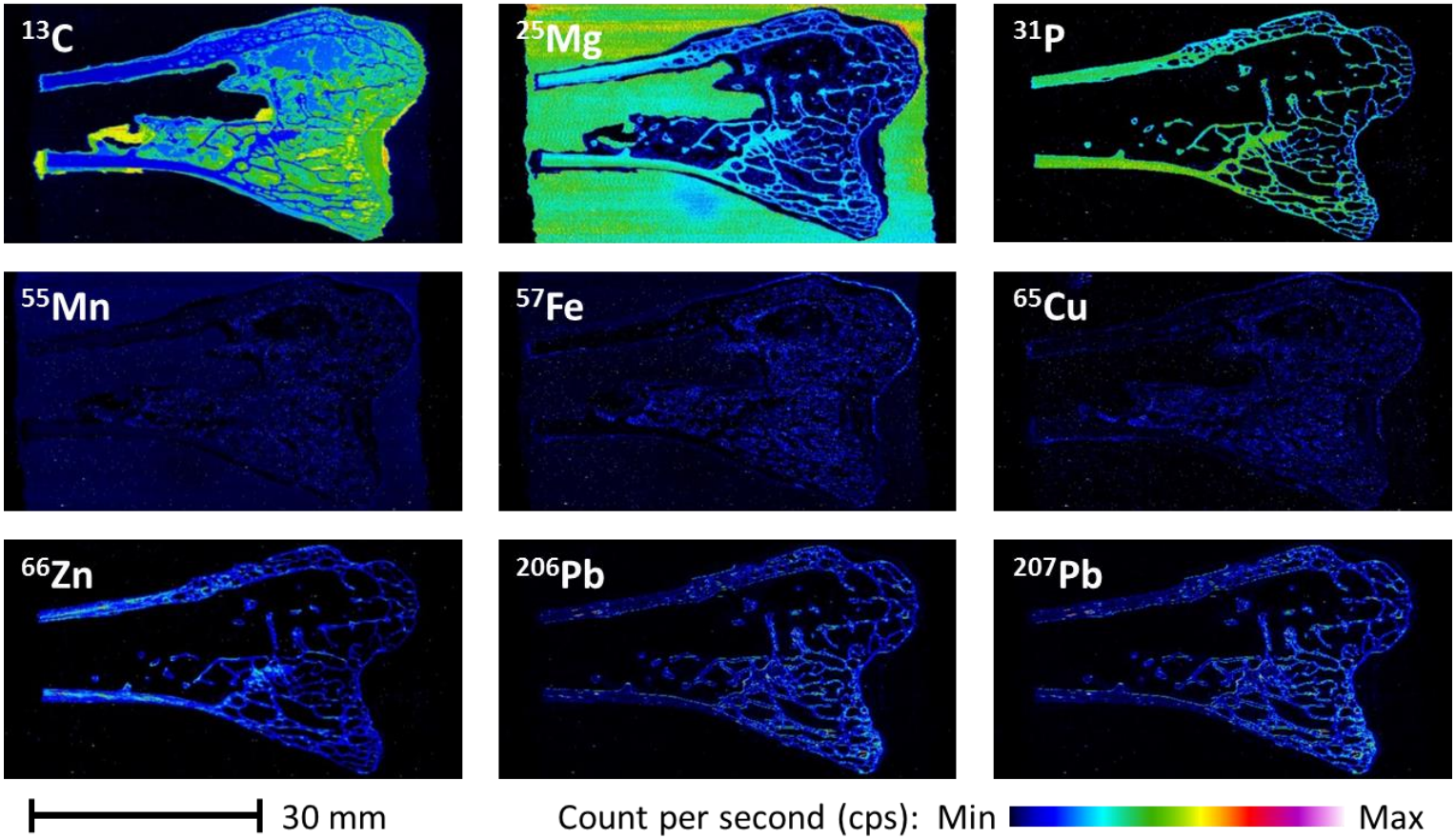
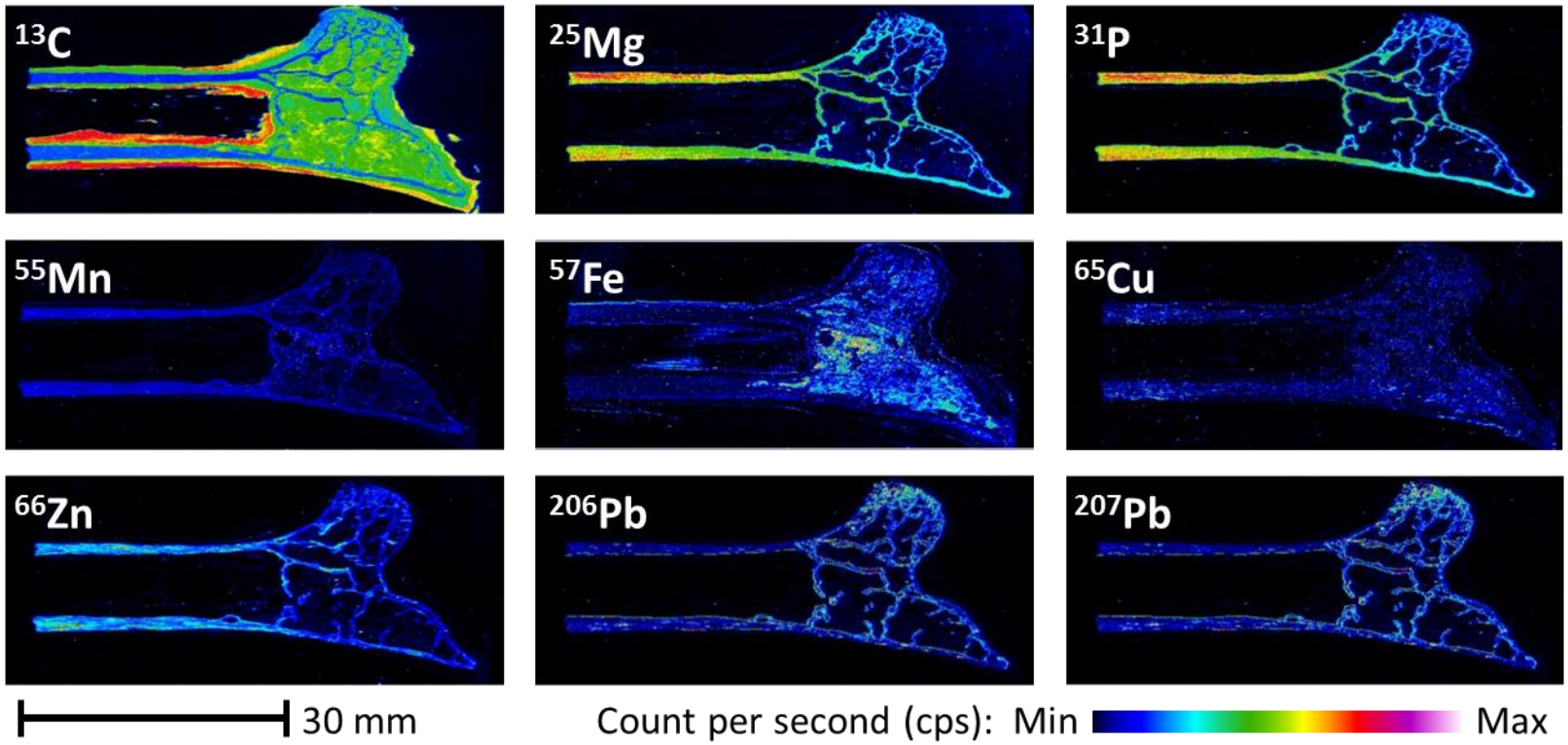


Fig. S10. Local distributions of ^{13}C, ^{25}Mg, ^{31}P, ^{55}Mn, ^{57}Fe, ^{65}Cu, ^{66}Zn, ^{206}Pb and ^{207}Pb in tibiotarsus of whooper swan. Scale bars are 30 mm. The ranges of count per second (cps) of metals differ depending on the images.



Note 4-1

Pb accumulation in tibiotarsus of Pb exposed chicken

Background and Objective

From the results of chapter 4, Pb concentration was completely different depending on the bone types in both eagles and swans. The hepatic concentration suggested that they were exposed to Pb acutely. However, the actual situation of Pb exposure was unclear. To confirm this tendency of Pb accumulation in bone as for the acute exposure, Pb concentration in chickens that had injected Pb and stored in our laboratory was measured.

Materials and Methods

In vivo *experiment using the broiler chicken (*Gallus gallus domesticus*)*

This *in vivo* experiment was carried out in Zambia for the SATREPS (Science and Technology Research Partnership for Sustainable Development) project by JST (Japan Science and Technology Agancy)/JICA (Japan International Cooperation Agency), and they provided specimens of tibiotarsus for my research. The animal experiment was performed under the supervision and with the approval of the Institutional Animal Care and Use Committee of Hokkaido University, Japan (approval number: 14-0119).

Male broiler chickens (n = 16, body weight: 2.0−3.5 kg) were provided by local farms in Zambia. They were acclimatized to the laboratory environment with a 12:12-hour light-dark cycle and given food and water *ad libitum* for one week before the commencement of the experiment at the School of Veterinary Medicine, University of

Zambia, Lusaka, Zambia. After acclimation, they were randomly assigned to four groups and injected with either saline or Pb (II) acetate trihydrate (Wako Pure Chemicals Industries) in the pectoral muscle. The following groups were considered: control group of injected saline; low dose group of Pb (II) acetate trihydrate (0.1 mg/kg body weight); middle dose group of Pb (II) acetate trihydrate (1 mg/kg body weight); and high dose group of Pb (II) acetate trihydrate (10 mg/kg body weight) ($n = 4$ in each group). 24 hours after the intramuscular injection, the chickens were euthanized and tibiotarsus was collected. Samples were preserved at -20 °C and transported to the Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan for analysis of Pb concentration. Before the analysis, diaphysis, epiphysis, and bone marrow were collected from the tibiotarsus.

Pb analysis

An analysis of Pb followed the protocol described in chapter 4.

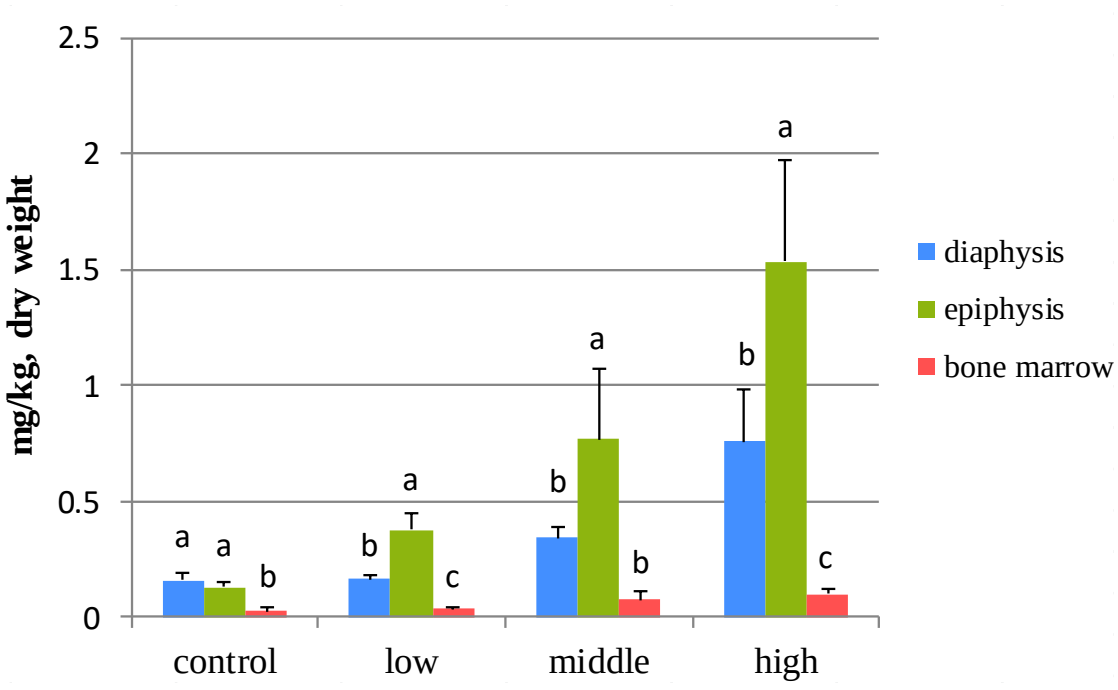
Statistics

For comparing Pb concentration among the four groups of chickens, data were analyzed using a Tukey-Kramer HSD (honest significant difference) test with a significance level of $p < 0.05$. Statistical analyses were performed in JMP Pro 11 (SAS Institute, Cary, NC, USA).

Results and Discussion

The accumulation pattern of Pb between cortical bone and trabecular bone was confirmed by the *in vivo* experiment of acute Pb exposure in chicken (Figure S5). Broiler chicken administrated Pb showed significantly higher Pb concentration in the epiphysis than that in the diaphysis 24 hours after the exposure (Tukey-Kramer HSD test, $p < 0.05$). Therefore, bone specimens that (1) mainly consist of trabecular bones and (2) contain bone marrow would immediately accumulate high levels of Pb.

Figure 1. Pb concentration in diaphysis, epiphysis, and bone marrow in the tibiotarsus of male broiler chicken (*n* = 16). Control group: injected saline, low dose group: 0.1 mg/kg Pb (II) acetate trihydrate, middle dose group: 1mg/kg Pb (II) acetate trihydrate, high dose group: 10mg/kg Pb (II) acetate trihydrate (n = 4 in each group). Markers of bones (not bone marrows) indicate the average of two specimens collected in the same places from each individual. Different letters in groups indicate significant differences among three parts (Tukey-Kramer HSD test, $p < 0.05$).



Note 4-2

Pb concentration in bones and organs of a white-tailed sea eagle (*Haliaeetus albicilla*) treated with chelation therapy

Background and Objective

One female white-tailed sea eagle (*Haliaeetus albicilla*) was found weakened in May 2016 in Hokkaido; she had a high level of Pb in her blood. Therefore, this eagle was treated with chelation therapy to reduce the Pb accumulated in her body. However, she unfortunately died in October 2016 even though her blood Pb level was decreasing (it was still at abnormal levels).

The results of chapter 4 showed that Pb accumulation in the bones of Pb-exposed birds was different depending on the bone type. In the current study, this eagle was treated with medicine for six months, and the Pb distribution in her bones might have changed during this period after the exposure. To confirm this, Pb concentrations in bones from throughout the entire body were measured, and Pb levels in the soft tissues of this eagle were also analyzed.

Materials and Methods

Sampling

One white-tailed sea eagle (adult, female) that died during the medical treatment was analyzed. Samples were provided from the Institute for Raptor Biomedicine Japan. As for the samples, bones (cranial bone, hyoid bone, atlas, axis, 3rd and 4th cervical vertebrae, notarium, scapula, sternum, keel, coracoideum, costa vertebralis, costa sternalis, ilium, pygostyle, humerus, radius, ulna, phalanx distalis digiti majoris, femur,

patella, tibiotarsus, fibula, tarsometatarsus, and os digiti pedis), spinal cord specimens, and bone marrow (axis, radius, ulna, tibiotarsus, and tarsometatarsus) were collected from the birds (Figure 1). For the vertebrae, the spinous process was collected. For the long bones, diaphysis was selected. Soft tissues (liver, kidney, brain, spleen, bile, ovary, oviduct, lung, pancreas, intestine, urine, thyroid gland) were also collected. Specimens were kept at -80°C until analysis.

Pb analysis

An analysis of Pb followed the protocol described in chapter 4.

Results and Discussion

The hyoid, axis, ilium, and patella accumulated higher Pb levels than the other bones. Chapter 4 showed that bone samples that (1) mainly consist of trabecular bone and (2) contain bone marrow could accumulate high levels of Pb after acute Pb exposure. The white-tailed sea eagle was treated with chelation therapy to reduce the Pb accumulation in her body, and a half-year had passed between the exposure and her death. Although the concentrations did not differ remarkably among bone types compared with the eagles in chapter 4, there was a same pattern that bone specimens that mainly consisted of trabecular bone and contained bone marrow showed higher accumulations than other bones. The results of the current study and chapter 4 indicated that Pb is first accumulated in trabecula bones because cell turnover is faster than in cortical bones, and cortical bone accumulates Pb gradually. Therefore, the comparison of Pb levels between trabecular and cortical bone could provide information about an individual's Pb exposure.

Regarding the organs, liver, kidney, and brain had higher concentrations than other organs. The presence of Pb in the brain showed that Pb can easily pass through the blood-brain barrier, and could remain there.

The main reason for the death of this eagle might not have been Pb poisoning itself. However, this study showed that Pb accumulated in many tissues, and can cause various problems in patients exposed to Pb.

Figure 1. The position of bone and bone marrow specimens collected from birds. The map of the skeleton diagram of bird was modified from "Squelette_oiseau.JPG: BIODIDAC derivative work: mario modesto (Squelette_oiseau.JPG)".

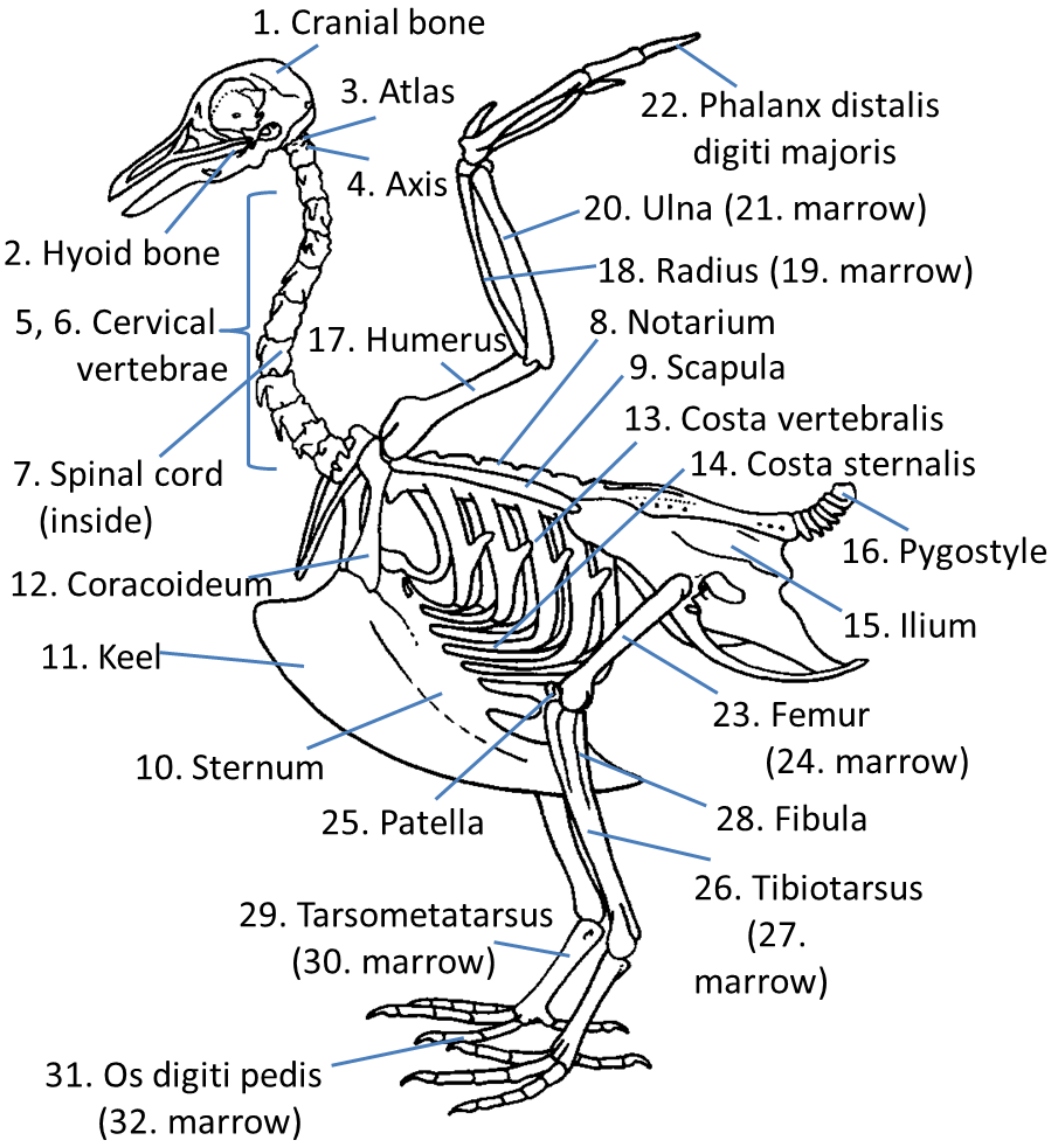


Figure 2. Pb distribution in specimens of bone and bone marrow from the white-tailed sea eagle. The position of each bone is shown in Figure 1 with the skeleton diagram of each bird.

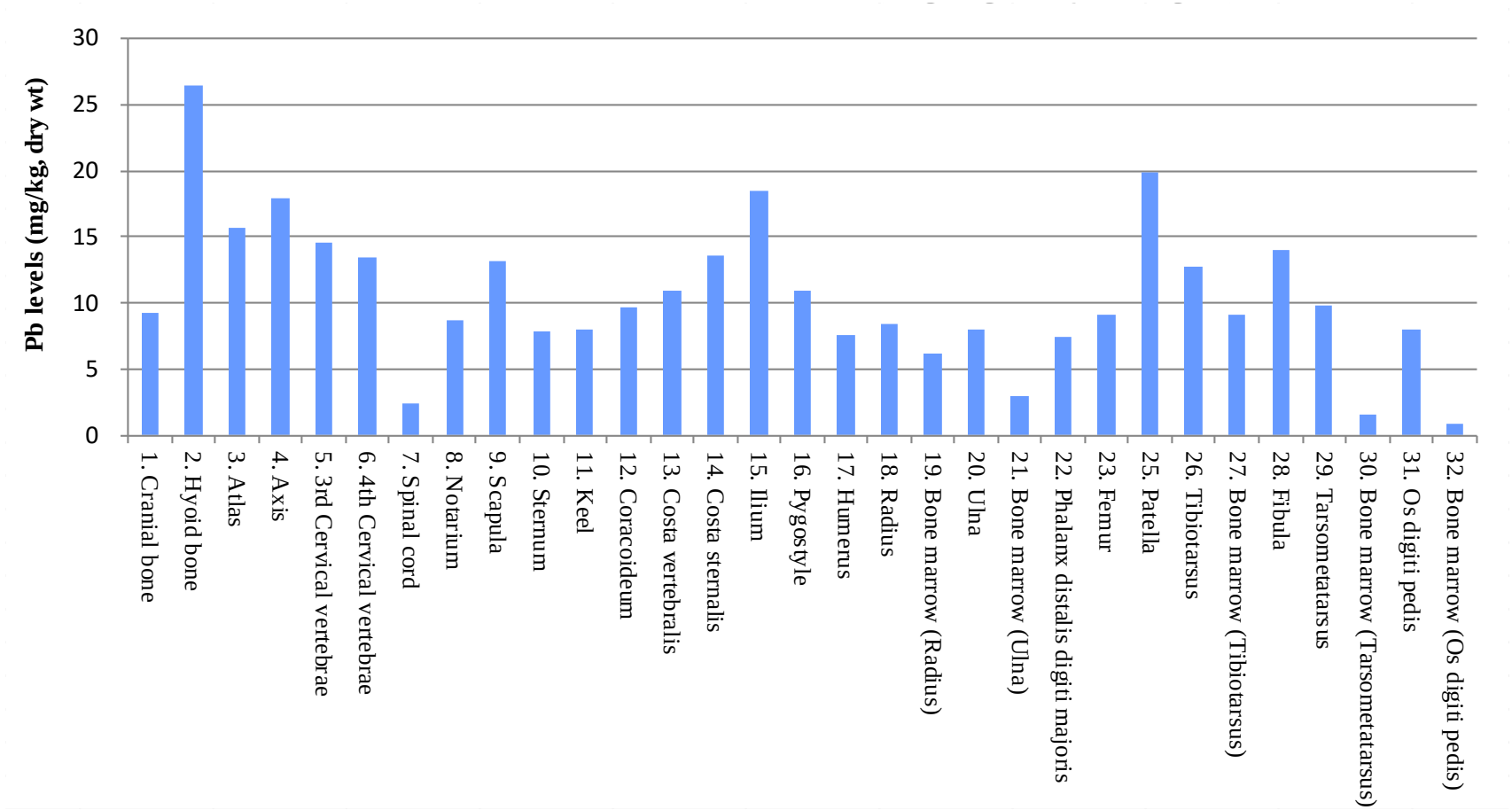
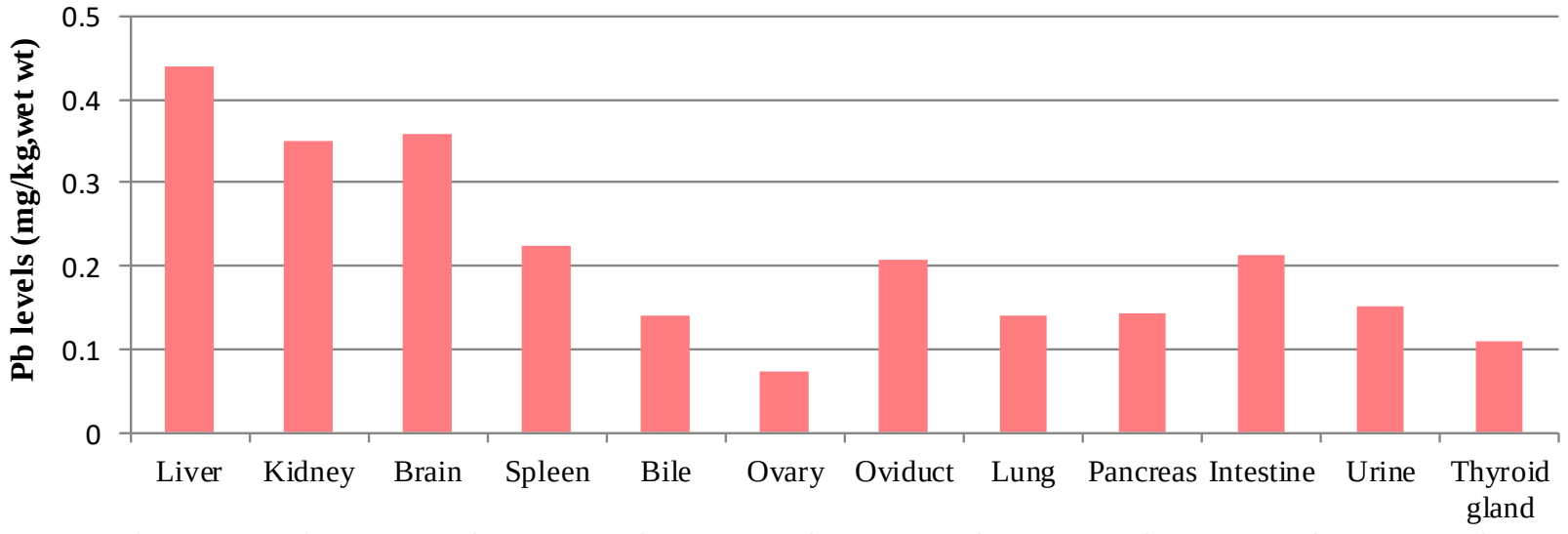


Figure 3. Pb concentration in organs, bile and urine.



Note 4-3

Pb concentration in bones of a white-fronted goose (*Anser albifrons frontalis*) poisoned by Pb

Background and Objective

One white-fronted goose (*Anser albifrons frontalis*) that likely died due to Pb poisoning, according to clinical signs and hepatic Pb level, was collected in Hokkaido. To compare with the data of chapter 4, Pb concentrations in the bones of this goose were measured.

Materials and Methods

Sampling

A white-fronted goose (*Anser albifrons frontalis*) that likely died due to Pb poisoning was provided by the Hokkaido Institute of Public Health. Bones (cranial bone, hyoid bone, atlas, axis, 3rd and 4th cervical vertebrae, notarium, sternum, keel, coracoideum, costa vertebralis, and costa sternalis) and spinal cord specimens were collected from the bird (Figure 1). Two samples were collected from same parts of all bone types, and the average concentration of the two specimens was used. Bone specimens were kept at -20°C.

Pb analysis

An analysis of Pb followed the protocol described in chapter 4.

Results and Discussion

Pb concentrations were different depending on the bone type (Figure 2), although the differences were small. This distribution pattern was similar to that of the swan (B) in chapter 4, but compared to the swans and eagles in chapter 4, Pb levels were low. Although it is difficult to guess the type of Pb exposure from these results, this goose might have died due to sub-acute or sub-chronic exposure to Pb. This is because the Pb concentration was high, but not remarkably so compared to the poisoned birds in chapter 4, and the Pb concentration did not differ much among bones. In the case of acute exposure, Pb concentrations in trabecular bone would much be higher than that in cortical bone. To refine the assessment of Pb exposure from the information provided by bones, further research is needed.

Figure 1. The position of bone and bone marrow specimens collected from birds. The map of the skeleton diagram of bird was modified from "Squelette_oiseau.JPG: BIODIDAC derivative work: mario modesto (Squelette_oiseau.JPG)".

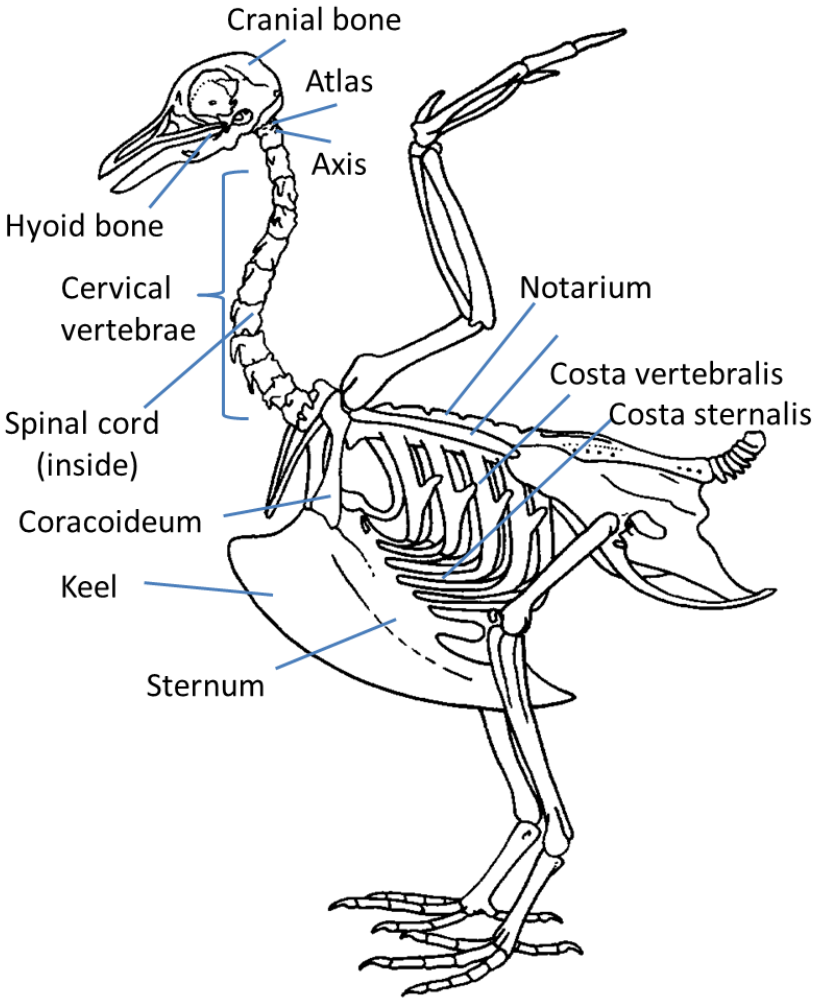
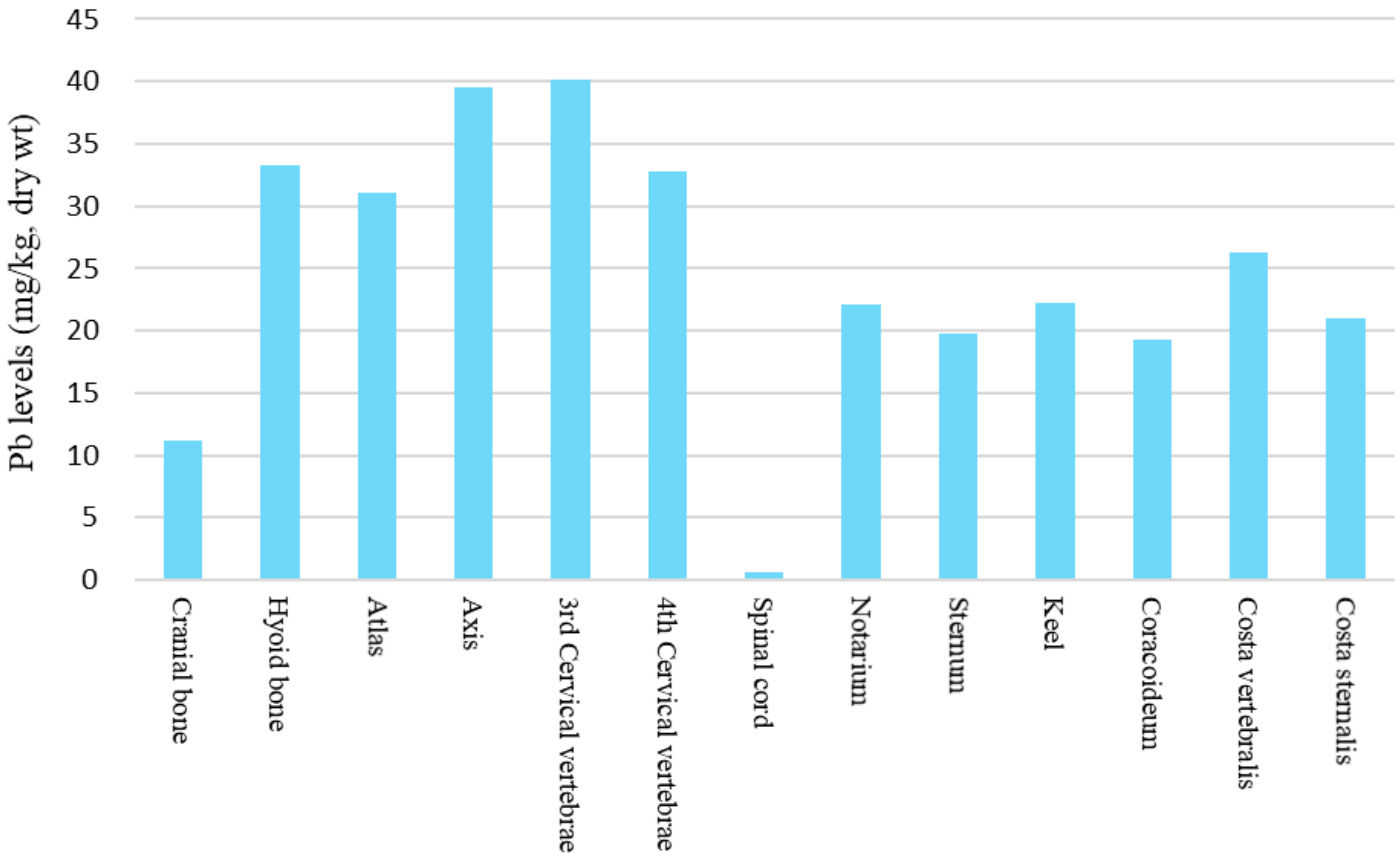


Figure 2.


Pb levels (mg/kg, dry wt)
45
40
35
30
25
20
15
10
5
0
Cranial bone
Hyoid bone
Atlas
Axis
3rd Cervical vertebrae
4th Cervical vertebrae
Spinal cord
Notarium
Sternum
Keel
Coracoideum
Costa vertebralis
Costa sternalis

CHAPTER 5

Discovery of novel renal biomarkers in a chicken model using a glycomic approach

Abstract

Avian species have a unique renal structure and abundant blood flow into the kidneys. Although many birds die due to nephrotoxicity caused by chemicals, there are no early biomarkers for renal lesions. Uric acid level in blood, which is generally used as a renal biomarker, is altered when the kidney function is damaged by over 70%. Therefore, early biomarkers for kidney injury in birds are needed. In humans, glycomics has been at the forefront of biological and medical sciences, and glycans are used as biomarkers of diseases, such as carcinoma. In this study, a glycomics approach was used to screen for renal biomarkers in chicken. First, a chicken model of kidney damage was generated by injection of diclofenac or cisplatin, which cause acute interstitial nephritis (AIN) and acute interstitial nephritis (ATN), respectively. The nephrotoxicity levels were determined by blood chemical test and histopathological analysis. The plasma *N*-glycans were then analyzed to discover renal biomarkers in birds. Levels of 14 glycans increased between pre- and post-administration in kidney-damaged chickens in the diclofenac group, and some of these glycans had the same presumptive composition as those in human renal carcinoma patients. Glycan levels did not change remarkably in the cisplatin group. It is possible that there are changes in glycan expression due to AIN but they do not reflect ATN. Although further research is needed in other species of birds, glycans are potentially useful biomarkers for AIN in avian species.

Key words: acute interstitial nephritis (AIN), acute tubular necrosis (ATN), bird, glycan, renal biomarker

Highlights

- Regarding the creating kidney-damaged model chicken, diclofenac caused AIN (acute interstitial nephritis) and cisplatin induced ATN (acute tubular necrosis).
- Glycans have the potential to be useful biomarkers for AIN but do not reflect ATN in avian species.

1. Introduction

Avian species have a unique kidney structure with abundant blood flow into the kidneys (Harr, 2002; Shideman et al., 1981) because of the renal portal veins. This system does not exist in mammals (Lierz, 2003), and the avian kidney is vulnerable to various chemicals from the blood.

Indeed, many birds die due to nephrotoxicity caused by chemicals. In the Indian subcontinent, over 95% of three vulture species were killed by the non-steroidal anti-inflammatory drug (NSAID), diclofenac (Green et al., 2004; Swan et al., 2006). Diclofenac was used for the medical treatment of cattle, and this medicine was accidentally ingested by vultures when they consumed cattle carcasses (Oaks et al., 2004). It has been reported that primary cultures of avian kidney cells were much more susceptible to diclofenac than mammalian cell cultures (Naidoo and Swan, 2009).

In addition, other NSAIDs, such as ketoprofen, cause renal lesions in birds as a side effect (Mohan et al., 2012; Mulcahy et al., 2003). Furthermore, lead (Pb), cadmium (Cd), mercury (Hg), and other therapeutic agents, such as anticancer drugs and antifungal agents, cause renal toxicity in birds (Filippich et al., 2001; Johnson, 1998; Joseph, 2000; Wolfe et al., 1998). In the case of humans, drug-induced kidney injury is a serious problem in clinical practice and account for 19% – 26% of cases of acute kidney injury (AKI) among hospitalized patients (Hosohata, 2016; Mehta et al., 2004). In avian species, there have been many reports of renal damage in both wild birds and companion birds.

The diagnosis of kidney disease in birds is challenging. Generally, uric acid (UA) level in blood is used as a renal biomarker in birds. However, UA is not an early biomarker because its levels can be altered when the kidney function is damaged by > 70% (Lierz, 2003). The end product of protein metabolism in birds is UA, and because most of the

UA in the urine is in an insoluble form, it does not have an osmotic effect (Styles and Phalen, 1998). Furthermore, as most UA is secreted from the proximal tubules and not filtered, blood UA levels will not be affected by moderate changes in the glomerular filtration rate (GFR) (Styles and Phalen, 1998). Even in the event of extensive tubular disease, polyuria and resultant polydipsia and the resulting increase in glomerular filtration can maintain UA levels within the normal range. Therefore, UA concentrations may not reflect glomerular disease and widespread tubular disease may also be present long before UA levels rise above normal.

Diagnosis of kidney disease is further complicated in that it is difficult to obtain urine samples from birds because the ureter opens into the cloaca, and the urine is stored in the cloaca or intestine until defecation of a semisolid mixture of urine and feces (Skadhauge, 1968). The level of phosphorus is not changed commonly in all species of birds although the concentration increases due to renal lesions in some avian species (Tully et al., 2009). Therefore, discovery and identification of novel biomarkers for kidney injury in birds are required.

Genomics and proteomics approaches are generally used for the discovery of biomarkers. Glycomics is also a useful tool to identify biomarkers (Adamczyk et al., 2012). Glycosylation is a frequent co-/posttranslational modification of proteins, which modulates a variety of biological functions (Dall'Olio et al., 2013). Glycan structures on newly synthesized glycoproteins are crucial for protein secretion (Moremen et al., 2012). Over 50% of proteins are glycosylated in humans, and the effects of disease states on glycan biosynthesis can be more evident than those on proteins (Adamczyk et al., 2012). It has been reported that glycans in humans may potentially be used as biomarkers of

renal carcinoma (Hatakeyama et al., 2014). Therefore, it is possible that glycans would be useful as biomarkers in birds.

The present study was performed to identify novel renal biomarkers in avian species for the conservation and to develop cures for wild birds as well as companion birds. First, a model of kidney damage was generated in chickens by injection of diclofenac or cisplatin, and renal biomarkers were examined. Acute kidney injury includes acute interstitial nephritis (AIN) and acute tubular necrosis (ATN) (Hosohata, 2016); diclofenac causes AIN, whereas cisplatin causes ATN. Diclofenac caused severe nephrotoxicity in birds as mentioned above, and the anticancer drug, cisplatin, induces renal lesions as a side effect and is used to make models of kidney injury in rats (Pabla and Dong, 2008; Pinches et al., 2012). The kidney shows greater accumulation of cisplatin than other organs, and the kidney is the major route for its excretion (Yao, X., Panichpisal, K., Kurtzman, 2007).

To our knowledge, this is the first study to use glycomics to discover biomarkers in birds. Plasma *N*-glycans in chicken were analyzed by glycoblotting, which can be used for high-throughput analysis of biological samples (Hirose et al., 2011), along with matrix-assisted laser desorption ionization, time-of-flight mass spectrometry (MALDI-TOF/MS), and they were profiled to discover novel biomarkers for kidney injury in avian species.

2. Materials and Methods

2.1. Animal Experiments

All experimental protocols were approved by the Laboratory Animal Care and Use Committee of Graduate School of Veterinary Medicine, Hokkaido University, Japan (approval number: 14-0119). The animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals, which conforms to the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The endpoints were weight loss > 20% compared to pre-injection, or severe clinical symptoms. Euthanasia was carried out by carbon dioxide inhalation under anesthesia by an overdose of isoflurane (Abbott Laboratories, Chicago, IL) after fasting for over 12 hours. The animals were monitored twice per day during the administration period to check their health. Their body weight was measured from pre-administration to the final day of the experiment. A 27 gauge (G) needle was used for injection to reduce pain and stress.

2.2. Experimental Design

Male white leghorn chickens (*Gallus gallus domesticus*) (n = 15, 10 weeks old, body weight: 1.2 – 1.4 kg) were purchased from Hokudo Co., Ltd. (Tokyo, Japan) and were housed under conditions of constant temperature (20°C ± 2°C) and humidity (40% ± 10%), with a 12:12-hour light:dark cycle and given food and water *ad libitum*. They were allowed to acclimatize to the environment for 1 week before commencement of the experiment. Animals were divided into the following four groups: (1) control group (injection of 20% DMSO, n = 3: Cont. -1, -2, -3); (2) diclofenac sodium group A (1.5 mg/kg body weight, n = 4: A-1, -2, -3, -4); (3) diclofenac sodium group B (2.0 mg/kg

body weight, $n = 4$: B-1, -2, -3, -4); and (4) cisplatin group (3.5 mg/kg body weight, $n = 4$: C-1,-2, -3, -4). Injection doses were considered according to reference papers of diclofenac treatments (Jain et al., 2009; Mohan et al., 2012; Naidoo et al., 2007) or cisplatin administrations (Cacini and Fink, 1995; Filippich et al., 2001).

Pre-administration plasma was collected 1 week after arrival. Administration was started after a further 1 week. The control group received injection of 20% dimethyl sulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan) diluted in saline once daily in the morning for four consecutive days. The treatment groups received administration of diclofenac sodium diluted in 20% DMSO into the pectoral muscle once daily in the morning for four consecutive days, or a single dose of cisplatin (Wako Pure Chemical Industries, Osaka, Japan) diluted in saline into the basilic vein. Control and diclofenac groups were euthanized on day 5 after the first administration, whereas the cisplatin group was euthanized on day 3 because of the endpoint of clinical signs.

2.3. Blood Collection

Blood samples (6 mL) were collected in the morning before administration and every day after the injection from the basilic vein using a 23 G or 24 G needle and heparin-containing syringe. Whole blood was stored on ice after collection and plasma was prepared by centrifugation within 2 hours after collection. Centrifugation was performed at 1630 × *g* for 20 minutes at 4°C. Plasma specimens were immediately frozen in liquid nitrogen and stored at –80°C.

2.4. Tissue Sample Collection

Chickens were euthanized with an overdose of isoflurane (Abbott Laboratories) and carbon dioxide. After euthanasia, the kidneys, liver, lungs, and heart were collected. The weights of the whole body, liver, and kidney were measured. The excised tissues were cut into small pieces and stored in 10% neutral buffered formalin for histopathological analysis.

2.5. Blood Tests

Aspartate aminotransferase (AST), total plasma protein (TPP), lactate dehydrogenase (LDH), creatine phosphokinase (CK), inorganic phosphate (P), and calcium (Ca) levels were determined using COBAS Ready® (Roche Diagnostics K.K., Basel, Switzerland) or DRI-CHEM 7000V (Fujifilm Co., Tokyo, Japan). The upper detection limit of UA by COBAS is 20 mg/dL, and UA in birds with high degrees of kidney damage exceeds this level. Therefore, UA level was analyzed by high-performance liquid chromatography separation and ultraviolet detection (HPLC-UV, 20A series; Shimadzu, Kyoto, Japan). The significance of each test is shown in the Supporting Information (Text S1 in Supporting Information).

For measurement of hematocrit (Ht), 200 μL of whole blood was collected from the basilic vein before dissection using a 24 G needle without heparin and immediately moved into tubes containing ethylenediaminetetraacetic acid (EDTA). Ht level was measured using a microhematocrit centrifuge (Kubota 3220; Kubota Corporation, Tokyo, Japan) at 15000 × *g* for 5 minutes.

2.6. Uric Acid Analysis by HPLC

Analyses were performed by HPLC-UV (20A series; Shimadzu) and the improved HPLC method of the Japan Society of Clinical Chemistry (JSCC) was used for measurement of UA. Briefly, 25-μL aliquots of plasma specimens or standard solution were mixed with 225 μL of 0.3 mol/L perchloric acid and cooled on ice for 30 minutes. The samples were mixed again by vortexing and centrifuged at 750 × *g* for 10 minutes at 4°C. Aliquots of 150 μL of the supernatants were collected and centrifuged again at 750 × *g* for 10 minutes at 4°C. Then, 50-μL aliquots of the supernatants were collected and moved to HPLC vials, followed by addition of 50 μL of 10 mmol/L ammonium acetate. For HPLC calibration, 100.3 mg of uric acid (Wako Pure Chemical Industries) was dissolved in 0.01 mol/L lithium carbonate in a final volume of 100 mL (1 g/L). Standard solutions (1.25, 2.5, 5, 10, 25 and 50 mg/L) were diluted with deionized distilled water. A UV detector set at 284 nm was used to monitor the effluent. Mobile phase A consisted of 10 mmol/L ammonium acetate (pH 4.8) and phase B consisted of 100% methanol. An Inertsil ODS-3 column (2.1 mm × 150 mm: GL Sciences, Inc., Tokyo, Japan) was used for separation at a flow rate of 0.2 mL/min and the injection volume was 5 μL. The R^2 value of the linear regression line was 0.998.

2.7. Histopathological Analysis

Paraffin-embedded kidney sections were stained with periodic acid-Schiff, and liver, heart, and lung sections were stained with hematoxylin and eosin.

2.8. Glycoblotting-based Plasma Glycomics

Analyses of *N*-glycans were performed according to the methods of Kamiyama et al. (2013). Plasma specimens were pretreated for release of *N*-glycans and subjected

to glycoblotting for enrichment and quantification of *N*-glycans prior to MALDI-TOF/MS. Briefly, pretreatment of plasma glycoproteins was performed to release whole *N*-glycans using Peptide-N-Glycosidase F (PNGase F) (New England BioLabs, Ipswich, MA). Glycans were selectively captured by glycoblotting using BlotGlyco® beads. Methyl esterification of sialic acid residues and transiminization reaction to tag *N*-glycans with benzyloxyamine (BOA) were carried out on the beads. BOA-tagged *N*-glycans were subjected to MALDI-TOF/MS analysis. More details regarding pretreatment, glycoblotting, mass spectrometry, and data analysis are presented in S2 Text. Expression levels of each glycan were expressed by the ratio with the plasma mixture of healthy chickens.

2.9. Statistical Analysis

To compare the results of biochemical analyses between pre- and post-administration, the weight of the body and tissues between controls and treatment groups were analyzed by Steel's test. For comparison of renal damage scores and glycan levels among chickens, data were analyzed using Spearman's rank correlation coefficient. Statistical analyses were performed using JMP Pro 13 (SAS Institute, Cary, NC). In all analyses, $p < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. Clinical Signs

In the diclofenac-treated group, chicken B-1 showed depressed activity and polyuria from the 2nd day of administration, and was dead on the 3rd day. Chicken A-1 also showed weakness from the 4th day. All four cisplatin-treated chickens had polyuria and depressed activity from the 2nd day. The other chickens generally appeared normal. The control group did not show any symptoms.

3.2. Biochemical Analysis

The plasma concentrations of UA, P, Ca, AST, LDH, CK, and TPP as indicators of various tissues are shown in Table 1. The normal UA concentration in chickens ranges from 2.5 to 8.1 mg/dL (Miller and Fowler, 2014). Although there were no significant differences between pre- and post-administration in any of the groups, several chickens in the treatment groups showed high levels of UA. In the diclofenac group, chicken A-1 exceeded the normal UA level 72 hours after injection. Chickens B-1 and B-2 showed high levels of UA after 24 hours, and the level in chicken B-4 increased after 72 hours. However, the UA level in chickens B-2 and B-4 recovered after 96 hours. In the cisplatin group, UA level was high in all chickens 48 hours after the injection, and markedly exceeded the reference level. As other indicators of renal lesions, P concentration was also elevated in some of these chickens; the reference level in chicken is 6.2 – 7.9 mg/dL (Miller and Fowler, 2014). Although AST level of chickens in the treatment group increased, the levels were within the normal range for turkeys of 255 – 499 IU/L (Miller and Fowler, 2014). The LDH level exceeded the reference level for turkeys of 420 – 1338 IU/L (Miller and Fowler, 2014).

3.3. Gross Pathology

All cisplatin-treated chickens, and chickens A-1, B-1, and B-2 in the diclofenac group showed pale kidneys compared with the controls (Fig. 1). Kidneys in these chickens were enlarged although there were no significant differences in kidney weight between controls and each exposure group (Table S1). The control group and the other chickens in the treatment groups did not show any gross pathological changes. The liver specimens in all chickens had no lesions and there were no significant differences in tissue weight (Table S1).

3.4. Histopathological Analysis

All cisplatin-treated chickens and four diclofenac-treated chickens showed renal damage (Fig. 2). Although they commonly showed degenerative and necrotic lesions in the proximal and distal tubules, and sometimes glomeruli, and the shape of nuclei in the proximal tubules became unclear, the histology was different between diclofenac- and cisplatin group. Diclofenac-treated chickens showed the infiltration of leukocytes such as heterophils in interstitium. In the cisplatin group, necrosis of tubules was shown and many proteinaceous casts in the tubular lumen were also found. In addition, tubular epithelial cells were detached from the basement membrane of some tubules in chickens A-1 and B-1 in the diclofenac group and all cisplatin-treated chickens. Chickens B-2 and B-4 in the diclofenac group showed mild degenerative lesions, such as slight dilation of proximal and distal tubular lumens. Chickens A-2, A-3, A-4, and B-3 appeared normal.

According to the histopathological changes, renal lesions were given scores from K0 (no lesions) to K5 (most severe). For scoring, the ratio of outer/lumen area at cross

section of tubules was measured using Axiovision Rel 4.8 software (Zeiss, Germany). In addition, three stages of histopathological alterations of kidney (Salamat et al., 2014) were used as a reference. Damage scores are as follows: K0, no lesions (the median of outer/lumen area was > 10); K1, mild damage, such as infiltration of heterophils and cells in the proximal tubular lumens in a very limited area (the median of outer/lumen area was 8-9); K2, moderate damage, such as infiltration of heterophils and cells in proximal tubular lumens, and dilation of distal tubular lumens in a large area (the median of outer/lumen area was 5-7); K3, severe damage, such as infiltration of heterophils and cells in the proximal tubular lumens, dilation of tubular lumens, and proteinaceous casts (the median of outer/lumen area was approximately 4); K4, severe damage with unclear structure of renal tubules and glomeruli, and several proteinaceous casts (the median of outer/lumen area was approximately 3); or K5, severe damage with many necrotic cells, and proteinaceous casts (the median of outer/lumen area was 1-2 and most of tubules were disintegrated). The damage levels of each chicken were as follows: K0 (control); K1 (B-4); K2 (B-2); K3 (C-2); K4 (A-1, C-4); K5 (B-1, C-1, C-3). In the exposure group, chickens A-2, A-3, A-4, and B-3 did not show histopathological changes, and they were ranked K0.

The livers in chicken B-1 and all cisplatin-treated chickens showed mild hyperemia (Fig. S1). There were no lesions in the heart or lung in any of the chickens, and the control group did not show histopathological changes in any tissues.

3.5. Glycomics Analysis

For glycomics analysis, 10 chickens (Cont.-1, -2, A-1, B-1, B-2, B-4, C-1, -2, -3, and -4) were selected according to the results of histopathological analysis and

biochemical analysis, and plasma *N*-glycans were measured. A total of 40 plasma *N*-glycans were detected, and the levels of each glycan are shown by the ratio with glycan expression of plasma mix from the controls and pre-injection chickens (Table S2). The control group did not show any significant differences due to injection. Fourteen glycans were increased in the diclofenac-treated kidney damaged chickens (Fig. 3, Table S3).

In the cisplatin group, glycan levels did not change significantly according to kidney injury, although the renal lesions were severe and UA concentrations were high.

In chickens A-1 and B-4, UA levels increased several days after the first injection, although the final concentrations were different between these two chickens. They exceeded the reference range of UA (2.5 – 8.1 mg/dL) 72 hours after the first exposure. For detection of earlier biomarkers than UA, the glycan expression levels before the increase in UA level were measured as an additional experiment. This additional study indicated that the levels of three glycans (ID No. 24, 33, and 38) increased earlier than UA (Table S4).

4. Discussion

In the present study, although there were individual differences, both cisplatin (3.5 mg/kg) and diclofenac (1.5 – 2.0 mg/kg) caused nephrotoxicity in chickens. The results of biochemical analyses indicated that UA, P, and LDH levels exceeded the respective normal ranges in several chickens. Both UA and P indicate severe renal lesions in chickens, but UA level was altered earlier than P level. According to the UA level, kidney function in the cisplatin group (C-1, -2, -3, and -4) would be markedly damaged 48 hours after injection. In the diclofenac group, chicken A-1 had severe renal injury from 72 hours after injection. The kidneys of chickens B-1 and B-2 were impaired with heavy renal failure after 24 hours, and chicken B-1 died due to nephrotoxicity. Although LDH level increased in the treatment groups, it was difficult to identify the cause, because elevation of LDH is nonspecific, and is found in skeletal and cardiac muscle, liver, kidney, bone, and erythrocytes (Tully et al., 2009).

Histopathological analysis of the kidney, liver, heart, and lung showed that chickens A-1, B-1, B-2, and B-4 and all cisplatin-treated chickens had damage almost specific to the kidney. Furthermore, the histology of kidney might indicate that diclofenac caused AIN and cisplatin induced ATN in chicken, although the infiltration of leukocytes in interstitial in diclofenac-treated chickens was not so severe compared to AIN in human. In the case of diclofenac exposure, renal damage levels were completely different depending on the individual, and this tendency was also described in the reports mentioned above. There may be large differences in sensitivity even within a species. Therefore, the renal damage was shown by scores for comparison with other analyses.

Glycomics analysis showed that a high degree of kidney damage was associated with increased levels of both sialylated and non-fucosylated glycans in diclofenac-treated

chickens. Although not applicable to six glycans (No. 9, 22, 24, 26, 34, and 37), this tendency was seen for the remaining 34 glycans. The synthesis pathway of *N*-linked glycans starts from the cytosolic surface of the endoplasmic reticulum membrane by addition of sugars, and in the early secretory pathway, the glycans have important roles in protein folding, oligomerization, quality control, sorting, and transport (Helenius and Aebi, 2001). The glycans acquire more complex structures and new functions in the Golgi complex (Helenius and Aebi, 2001). The *trans* compartment elaborates additional branching and capping reactions on complex *N*-glycans, and capping reactions are continued in the *trans*-Golgi network (Moremen et al., 2012). Therefore, the final synthesis pathway, where sialic acids are attached, could be disturbed, or glycoproteins that have these glycans may be susceptible to the effects of kidney injury.

Furthermore, from the results of additional measurements in chickens A-1 and B-4, No. 24, 33, and 38 glycan levels increased earlier than UA level. Therefore, these may be useful as early biomarkers for kidney injury caused by diclofenac in birds.

In cisplatin-treated chickens, there is no relation between glycan expression and kidney damage. Therefore, the glycans that increased in the diclofenac group may indicate the effects of diclofenac injection, rather than kidney injury itself. Another possibility is that glycans may have reflected parts of kidney injury, and not all types of renal damage.

With regard to AIN and ATN, although the mechanism underlying AIN is underestimated even in human, it is generally accepted that the pathogenesis is based on an immunologic reaction against endogenous nephritogenic antigens or exogenous antigens processed by tubular cells, with cell-mediated immunity having a major pathogenic role (Praga and González, 2010). In avian species, there is less information.

However, it is reported that diclofenac causes nephrotoxicity due to reduction of UA transport by interfering with p-aminohippuric acid (PAH) channels in the chicken (Naidoo and Swan, 2009). The mechanism of diclofenac-induced renal failure in oriental white backed vultures has been proposed to be through inhibition of the modulating effect of prostaglandin on angiotensin II-mediated adrenergic stimulation (Jain et al., 2009). Cisplatin induces DNA damage, either necrotic or apoptotic cell death, formation of reactive oxygen species, mitochondrial dysfunction, and caspase activation (Ramesh and Reeves, 2002). These events cause both acute and chronic kidney injury, and the nephrotoxicity is characterized by activation of both proinflammatory cytokines and chemokines (Havasi and Borkan, 2016).

5. Conclusions

The mechanisms of metabolism of diclofenac and cisplatin are different. Therefore, it is possible that glycans reflect the limited pathway of kidney injury, and glycan expression profiles change due to AIN but do not reflect ATN. Although further studies including investigation of species differences are needed, glycans have the potential to be useful biomarkers for AIN in avian species. The glycan expression profile may reflect some types of kidney injury, and these molecules have the potential for use as biomarkers for the evaluation of functional disorders in birds.

Table 1. Plasma concentrations of UA (uric acid), P (inorganic phosphate), Ca (calcium), AST (aspartate aminotransferase), LDH (lactate dehydrogenase), CK (creatine phosphokinase), and TPP (total plasma protein) in diclofenac- or cisplatin-treated and control chickens

		pre-	24 h	48 h	72 h	96 h
Cont. (n = 3)	UA	5.0 ± 0.5	3.2 ± 0.3	2.4 ± 1.0	2.7 ± 0.6	2.9 ± 0.6
	P	7.1 ± 0.5	6.2 ± 0.4	6.4 ± 0.6	6.1 ± 0.0	6.1 ± 0.9
	Ca	9.7 ± 0.5	9.6 ± 0.5	9.8 ± 0.5	10.1 ± 0.2	10.3 ± 0.5
	AST	132 ± 8	142 ± 5	168 ± 18	164 ± 17	149 ± 18
	LDH	706 ± 358	575 ± 164	639 ± 99	497 ± 48	514 ± 171
	CK	1110 ± 468	844 ± 107	1290 *[1]	1437 ± 191	514 ± 171
	TPP	2.9 ± 0.1	2.7 ± 0.3	2.9 ± 0.3	2.9 ± 0.3	2.6 ± 0.5
Diclofenac A (1.5 mg/kg, n = 4)	UA	4.4 ± 1.0	3.7 ± 1.0	3.8 ± 1.2	72.9 ± 117.8	48.8 ± 78.6
	P	7.5 ± 0.3	6.8 ± 0.7	7.0 ± 0.4	8.1 ± 1.4	8.8 ± 3.2
	Ca	9.9 ± 0.2	9.7 ± 0.2	9.3 ± 0.1	9.3 ± 0.2	9.1 ± 1.2
	AST	132 ± 5.9	198 ± 10.1	250 ± 16.1	304 ± 69.6	302 ± 88.1
	LDH	433 ± 43	1490 ± 234	1967 ± 639	1710 ± 1002	941 ± 202
	CK	1134 ± 284	>2000	>2000	>2000	>2000
	TPP	3.0 ± 0.3	2.9 ± 0.2	3.1 ± 0.2	3.1 ± 0.2	2.8 ± 0.3

Diclofenac B (2.0 mg/kg, n = 4)	UA	3.7 ± 0.5	52.6 ± 64.5	74.5 ± 103.9	12.0 ± 6.6 *3	6.7 ± 3.3 *3
	P	7.9 ± 0.7	7.2 ± 0.8	7.3 ± 0.9 *3	7.9 ± 1.2 *3	6.1 ± 1.1 *3
	Ca	9.8 ± 0.3	9.7 ± 0.1	9.2 ± 0.6	9.6 ± 0.4 *3	9.5 ± 0.2 *3
	AST	130 ± 14	228 ± 67	287 ± 22	282 ± 12 *3	258 ± 12 *3
	LDH	492 ± 132	1596 ± 672	2437 ± 978	2032 ± 984	985 ± 313
	CK	1340 ± 423	>2000	>2000	>2000	>2000
	TPP	2.9 ± 0.2	2.9 ± 0.3	2.9 ± 0.3	3.1 ± 0.6 *3	2.8 ± 0.4 *3
Cisplatin (3.5 mg/kg, n = 4)	UA	3.5 ± 0.3	8.0 ± 1.0	77.0 ± 16.2	—	—
	P	4.7 ± 0.4	6.3 ± 0.2	8.0 ± 1.0	—	—
	Ca	10.5 ± 0.5	10.9 ± 0.3	11.6 ± 1.5	—	—
	AST	137 ± 8	170 ± 8	246 ± 19	—	—
	LDH	425 ± 74	370 ± 71	870 ± 302	—	—
	CK	870 ± 69	1069 ± 70	1020 ± 169	—	—
	TPP	3.4 ± 0.2	3.8 ± 0.0	3.3 ± 0.6	—	—

* The values were calculated from one chicken*1, two chickens*2 or three chickens*3, because the others exceeded the detection limit (LDH: 3937 IU/L, CK: 2000 IU/L).

In diclofenac group B, one chicken died on the 3rd day of administration.

There were no significant differences between pre- and post-administration (Steel's test, $p < 0.05$).

Fig. 1. Gross pathological features of kidneys in diclofenac- or cisplatin-treated and control chickens

The chickens treated with diclofenac (a) or cisplatin (b) showed pale and enlarged kidneys compared with the controls (c). The kidney weights are shown in Table S2.

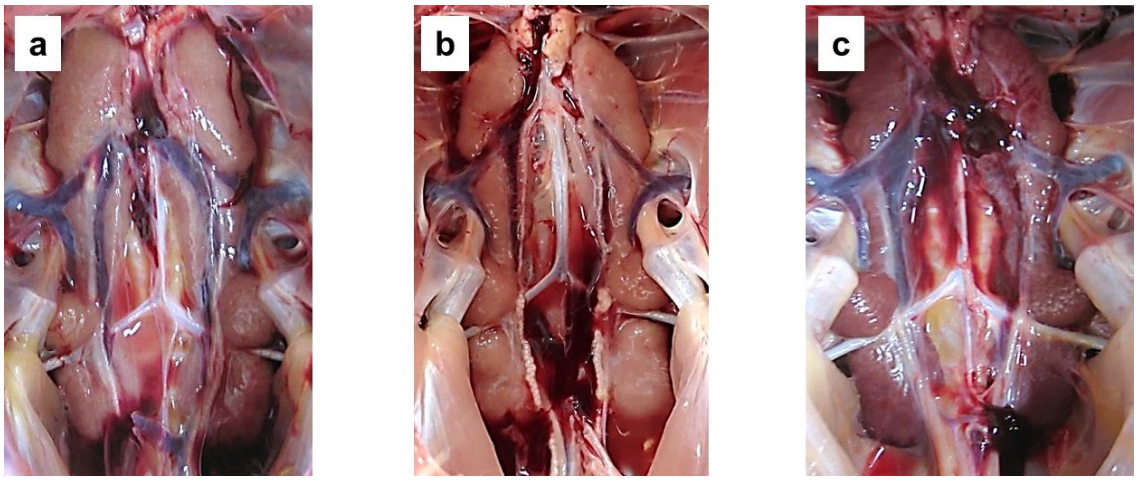


Fig. 2. Histopathological features of the kidneys in diclofenac- or cisplatin-treated and control chickens

Chickens treated with diclofenac (a) or cisplatin (b) showed severe renal lesions compared with controls (c). Briefly, there were degenerative and necrotic lesions, such as proteinaceous casts (arrowheads), heterophil infiltration into the tubulointerstitium and dead cells in the proximal tubular lumens (white arrows), and dilation of proximal and distal tubular lumens (black arrows). Diclofenac-treated chickens showed much infiltration of heterophils and cisplatin-treated chickens had a large number of proteinaceous casts. The controls showed normal renal structures.

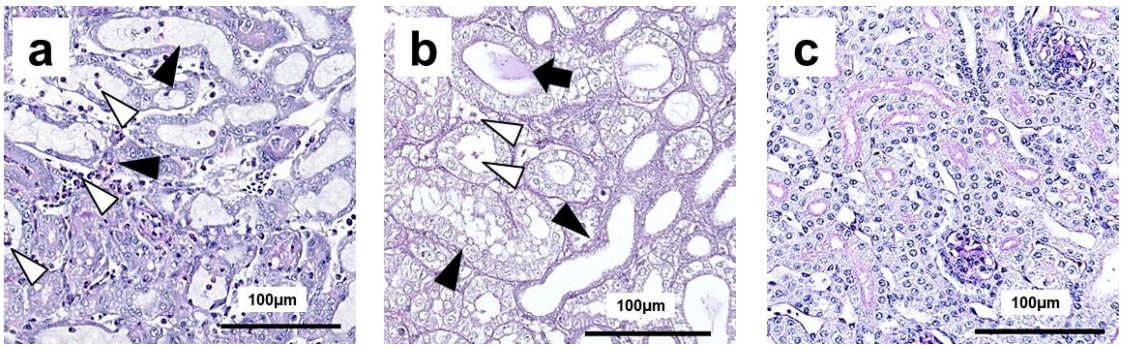
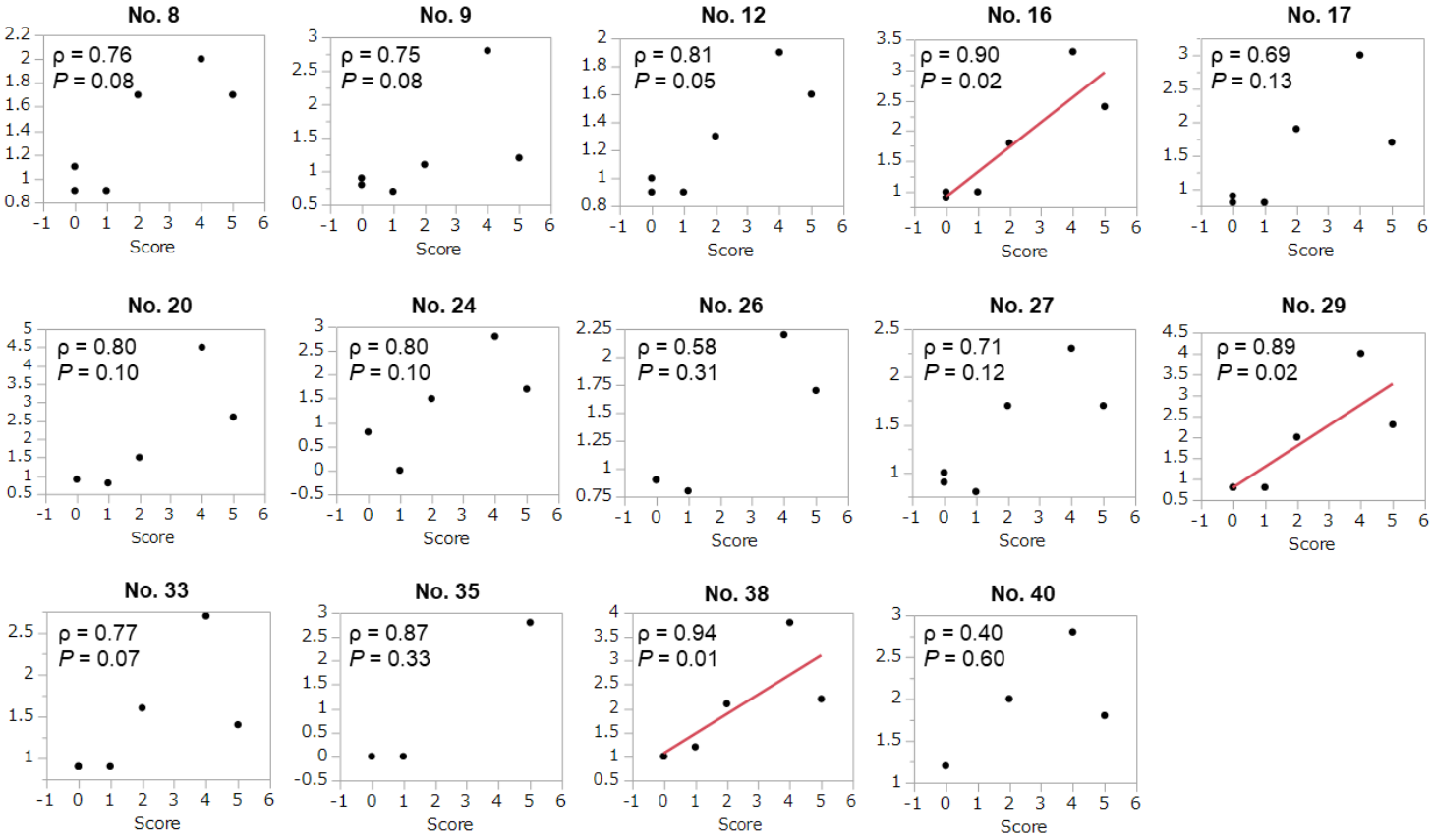


Fig. 3. Spearman's rank correlation coefficients of expression levels for 14 increased *N*-glycans (μM) on the final day and renal damage score in the diclofenac-treated group. No. 16, 29 and 38 had significant correlation and lines showed the linear approximation.



References

Adamczyk, B., Tharmalingam, T., Rudd, P.M., 2012. Glycans as cancer biomarkers. Biochim. Biophys. Acta (BBA)-General Subj. 1820, 1347–1353.

Cacini, W., Fink, I.M., 1995. Toxicity and excretion of cisplatin in the avian kidney. Comp. Biochem. Physiol. Part C Pharmacol. Toxicol. Endocrinol. 111, 343–350.

Dall'Olio, F., Vanhooren, V., Chen, C.C., Slagboom, P.E., Wuhrer, M., Franceschi, C., 2013. *N*-glycomic biomarkers of biological aging and longevity: a link with inflammaging. Ageing Res. Rev. 12, 685–698.

Filippich, L.J., Bucher, A.M., Charles, B.G., Sutton, R.H., 2001. Intravenous cisplatin administration in sulphur-crested cockatoos (*Cacatua galerita)*: Clinical and pathologic observations. J. Avian Med. Surg. 15, 23–30.

Green, R.E., Newton, I.A.N., Shultz, S., Cunningham, A.A., Gilbert, M., Pain, D.J., Prakash, V., 2004. Diclofenac poisoning as a cause of vulture population declines across the Indian subcontinent. J. Appl. Ecol. 41, 793–800.

Harr, K.E., 2002. Clinical chemistry of companion avian species: a review. Vet. Clin. Pathol. 31, 140–151.

Hatakeyama, S., Amano, M., Tobisawa, Y., Yoneyama, T., Tsuchiya, N., Habuchi, T., Nishimura, S.-I., Ohyama, C., 2014. Serum N-glycan alteration associated with renal cell carcinoma detected by high throughput glycan analysis. J. Urol. 191, 805–813.

Havasi, A., Borkan, S.C., 2016. Apoptosis and acute kidney injury. Kidney Int. 80, 29–40. doi:10.1038/ki.2011.120

Helenius, A., Aebi, and M., 2001. Intracellular Functions of N-Linked Glycans. Sci. 291, 2364–2369. doi:10.1126/science.291.5512.2364

Hirose, K., Amano, M., Hashimoto, R., Lee, Y.C., Nishimura, S.-I., 2011. Insight into glycan diversity and evolutionary lineage based on comparative avio-*N*-glycomics and sialic acid analysis of 88 egg whites of *Galloanserae*. Biochemistry 50, 4757–4774.

Hosohata, K., 2016. Role of oxidative stress in drug-induced kidney injury. Int. J. Mol. Sci. 17, 1826.

Jain, T., Koley, K.M., Vadlamudi, V.P., Ghosh, R.C., Roy, S., Tiwari, S., Sahu, U., 2009. Diclofenac-induced biochemical and histopathological changes in white leghorn birds (*Gallus domesticus*). Indian J. Pharmacol. 41, 237–241. doi:10.4103/0253-7613.58515

Johnson, F.M., 1998. The genetic effects of environmental lead. Mutat. Res. Mutat. Res. 410, 123–140.

Joseph, V., 2000. Aspergillosis in raptors, in: Seminars in Avian and Exotic Pet Medicine. Elsevier, pp. 66–74.

Kamiyama, T., Yokoo, H., Furukawa, J., Kurogochi, M., Togashi, T., Miura, N., Nakanishi, K., Kamachi, H., Kakisaka, T., Tsuruga, Y., 2013. Identification of novel serum biomarkers of hepatocellular carcinoma using glycomic analysis. Hepatology 57, 2314–2325.

Lierz, M., 2003. Avian renal disease: pathogenesis, diagnosis, and therapy. Vet. Clin. North Am. Exot. Anim. Pract. 6, 29–55.

Mehta, R.L., Pascual, M.T., Soroko, S., Savage, B.R., Himmelfarb, J., Ikizler, T.A., Paganini, E.P., Chertow, G.M., (PICARD, P. to I.C. in A.R.D., 2004. Spectrum of acute renal failure in the intensive care unit: the PICARD experience. Kidney Int. 66, 1613–1621.

Miller, R.E., Fowler, M.E., 2014. Fowler's zoo and wild animal medicine. Elsevier Health Sciences.

Mohan, K., Jayakumar, K., Narayanaswamy, H.D., Manafi, M., Pavithra, B.H., 2012. An initial safety assessment of hepatotoxic and nephrotoxic potential of intramuscular ketoprofen at single repetitive dose level in broiler chickens. Poult. Sci. 91, 1308–1314.

Moremen, K.W., Tiemeyer, M., Nairn, A. V, 2012. Vertebrate protein glycosylation: diversity, synthesis and function. Nat Rev Mol Cell Biol 13, 448–462.

Mulcahy, D.M., Tuomi, P., Larsen, R.S., 2003. Differential mortality of male spectacled eiders (*Somateria fischeri*) and king eiders (*Somateria spectabilis*) subsequent to anesthesia with propofol, bupivacaine, and ketoprofen. J. Avian Med. Surg. 17, 117–123.

Naidoo, V., Duncan, N., Bekker, L., Swan, G., 2007. Validating the domestic fowl as a model to investigate the pathophysiology of diclofenac in *Gyps* vultures. Environ. Toxicol. Pharmacol. 24, 260–266.

Naidoo, V., Swan, G.E., 2009. Diclofenac toxicity in *Gyps* vulture is associated with decreased uric acid excretion and not renal portal vasoconstriction. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 149, 269–274.

Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad, H.L., Ahmed, S., Iqbal Chaudhry, M.J., Arshad, M., Mahmood, S., Ali, A., Ahmed Khan, A., 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. Nature 427, 630–633.

Pabla, N., Dong, Z., 2008. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. Kidney Int. 73, 994–1007.

Pinches, M., Betts, C., Bickerton, S., Burdett, L., Thomas, H., Derbyshire, N., Jones, H.B., Moores, M., 2012. Evaluation of novel renal biomarkers with a cisplatin model of kidney injury: gender and dosage differences. Toxicol. Pathol. 40, 522–533.

Praga, M., González, E., 2010. Acute interstitial nephritis. Kidney Int. 77, 956–961.

Ramesh, G., Reeves, W.B., 2002. TNF-α mediates chemokine and cytokine expression and renal injury in cisplatin nephrotoxicity. J. Clin. Invest. 110, 835–842. doi:10.1172/JCI15606

Salamat, N., Etemadi-Deylami, E., Movahedinia, A., Mohammadi, Y., 2014. Heavy metals in selected tissues and histopathological changes in liver and kidney of common moorhen (*Gallinula chloropus*) from Anzali Wetland, the south Caspian Sea, Iran. Ecotoxicol. Environ. Saf. 110, 298–307.

Shideman, J.R., Evans, R.L., Bierer, D.W., Quebbemann, A.J., 1981. Renal venous portal contribution to PAH and uric acid clearance in the chicken. Am. J. Physiol. Physiol. 240, F46–F53.

Skadhauge, E., 1968. The cloacal storage of urine in the rooster. Comp. Biochem. Physiol. 24, 7–18.

Styles, D.K., Phalen, D.N., 1998. Clinical avian urology, in Seminars in Avian and Exotic Pet Medicine. WB Saunders. pp. 104–113.

Swan, G.E., Cuthbert, R., Quevedo, M., Green, R.E., Pain, D.J., Bartels, P., Cunningham, A.A., Duncan, N., Meharg, A.A., Oaks, J.L., 2006. Toxicity of diclofenac to Gyps vultures. Biol. Lett. 2, 279–282.

Tully, T.N., Dorrestein, G.M., Jones, A.K., 2009. Handbook of avian medicine. Elsevier/Saunders.

Wolfe, M.F., Schwarzbach, S., Sulaiman, R.A., 1998. Effects of mercury on wildlife: A comprehensive review. Environ. Toxicol. Chem. 17, 146–160. doi:10.1002/etc.5620170203

Yao, X., Panichpisal, K., Kurtzman, N., 2007. Cisplatin Nephrotoxicity: A Review. Am J Med Sci 334, 115–124.

Supplementary data

Text S1. Significance of each biochemical test

Text S2. Glycoblotting-based plasma glycomics.

Table S1. Weights of the body, liver, and kidney after exposure in chickens

Table S2. Ratios of all detected *N*-glycans (μM) on the final day/pre-administration

Table S3. Ratios of increased *N*-glycans (μM) on the final day/pre-administration in diclofenac-treated group

Table S4. Expression levels of glycans (No. 24, 33, and 38) and UA level in chickens A-1 (a) and B-4 (b) in which UA levels exceeded the normal range (2.5 – 8.1 mg/dL) after several days.

Fig. S1. Histopathological features of the liver in diclofenac- or cisplatin-treated and control chickens.

Text S1. Significance of each biochemical test

The plasma concentrations of UA, AST, CK, LDH, P, and Ca are indicators of various tissues as follows (Tully et al., 2009). AST activity is considered to be a very sensitive but nonspecific indicator of hepatocellular disease because muscular damage changes AST levels in avian species. CK elevation is associated with significant disruption of skeletal muscle, cardiac muscle, and nervous tissue. Generally, increased CK levels are compared with AST and LDH levels. Elevated AST without elevation of CK is highly suggestive of liver disruption and concurrent elevation of AST and CK suggests muscle disruption or concurrent damage to the liver and muscle or liver and nervous tissue. Elevation of LDH is nonspecific because it is found in skeletal and cardiac muscle, liver, kidney, bone, and erythrocytes, but LDH levels may follow the process of liver disease and change more quickly than AST levels. Elevated P levels resulting from renal disease suggest chronicity, although this is less common in bird species, and increases are also observed in hypoparathyroidism and nutritional secondary hyperparathyroidism. Blood Ca levels are directly linked to albumin levels, and dehydration sometimes causes elevation of albumin level, leading to an increase in blood Ca. Elevated levels of Ca are also associated with vitamin D3 toxicity, osteolytic bone tumors, and renal adenocarcinoma.

Text S2. Glycoblotting-based plasma glycomics.

Experimental Procedures: Plasma N-Glycomics by Glycoblotting

N-glycans from plasma samples were purified by glycoblotting using BlotGlyco®. These are commercially available synthetic polymer beads with high-density hydrazide groups (Sumitomo Bakelite, Tokyo, Japan). All procedures used the SweetBlot automated glycan purification system containing a 96-well plate platform (System Instruments, Tokyo, Japan).

Enzymatic Degradation of Plasma N-Glycans

Aliquots of 10 μL of plasma samples were dissolved in 50 μL of a 106 mM solution of ammonium bicarbonate containing 12 mM 1,4-dithiothreitol and 0.06% 1-propanesulfonic acid, 2-hydroxyl-3-myristamido (Wako Pure Chemical Industries, Osaka, Japan). After incubation at 60°C for 30 minutes, 123 mM iodoacetamide (10 μL) was added to the mixtures followed by incubation in the dark at room temperature to enable reductive alkylation. After 60 minutes, the mixture was treated with 200 U of trypsin (Sigma-Aldrich, St. Louis, MO) at 37°C for 2 hours, followed by heat inactivation of the enzyme at 90°C for 10 minutes. After cooling to room temperature, the *N*-glycans were released from the tryptic glycopeptides by incubation with 325 U of PNGase F (New England BioLabs) at 37°C for 6 hours.

N-Glycan Purification and Modification by Glycoblotting

Glycoblotting of sample mixtures containing whole plasma *N*-glycans was performed in accordance with previously described procedures. Commercially available BlotGlyco® beads (500 μL) (10 mg/mL suspension; Sumitomo Bakelite) were aliquoted

into the wells of a MultiScreen Solvinert hydrophilic polytetrafluoroethylene (PTFE) 96-well filter plate (EMD Millipore, Billerica, MA). After removal of the water using a vacuum pump, 20 μL of PNGase F-digested samples were applied to the wells, followed by the addition of 180 μL of 2% acetic acid in acetonitrile. The filter plate was then incubated at 80°C for 45 minutes to capture the *N*-glycans onto the beads by a chemically stable and reversible hydrazine bond. The beads were then washed using 200 μL of 2 M guanidine-HCl in 10 mM ammonium bicarbonate, followed by washing with the same volume of water and 1% triethyl amine in methanol. Each washing step was performed twice. The *N*-glycan linked beads were next incubated with 5% acetic anhydride in ethanol for 30 minutes at room temperature so that unreacted hydrazide groups would become capped by acetylation. After capping, the reaction solution was removed under vacuum and the beads were serially washed with 2 × 200 μL of 10 mM HCl, methanol, and dioxane as a pretreatment for sialic acid modification. On-bead methyl esterification of carboxyl groups in the sialic acids was carried out with 100 μL of 20 mM 3-methyl-1-P-tolyltriazene (Tokyo Chemical Industry, Tokyo, Japan) in dioxane at 60°C for 90 minutes to dryness. After methyl esterification of the more stable glycans, the beads were serially washed in 200 μL of dioxane, water, 1% triethylamine in methanol, and water. The captured glycans were then subjected to transiminization reaction with BOA (Tokyo Chemical Industry) for 45 minutes at 80°C. After this reaction, 150 μL of water was added to each well, followed by the recovery of derivatized glycans under vacuum.

MALDI-TOF and TOF/TOF Analysis

The *N*-glycans purified by glycoblotting were directly diluted with α-cyano-4-hydroxycinnamic acid diethylamine salt (Sigma-Aldrich) as ionic liquid matrix and

spotted onto the MALDI target plate. The analytes were then subjected to MALDI-TOF/MS analysis using an Ultraflex time-of-flight mass spectrometer III (Bruker Daltonics, Billerica, MA) in reflector, positive ion mode and typically summing 1000 shots. The *N*-glycan peaks in the MALDI-TOF/MS spectra were selected using FlexAnalysis v. 3 (Bruker Daltonics). The intensity of the isotopic peak of each glycan was normalized using 40 μM internal standard (A2 amide; Tokyo Chemical Industry) for each status, and its concentration was calculated from a calibration curve using human plasma standards. The glycan structures were estimated using the GlycoMod Tool (http://br.expasy.org/tools/glycomod/), so that our system could quantitatively measure 40 *N*-glycans.

Table S1. Weights of the body, liver, and kidney after exposure in chickens

In the case of body weight, the value of the day prior to euthanasia was used because they were fasted from the night of the previous day.

Group	Body weight (g)	Liver weight (g)	Kidney weight (g)	Liver/Body weight. (%)	Kidney/Body weight. (%)
Control	1489 ± 121	23.9 ± 0.6	9.5 ± 0.7	1.6 ± 0.1	0.6 ± 0.1
Diclofenac A (1.5 mg/kg)	1549 ± 41	25.6 ± 3.9	13.1 ± 4.4	1.6 ± 0.2	0.8 ± 0.3
Diclofenac B (2.0 mg/kg)	1455 ± 110	26.5 ± 5.5	14.2 ± 5.9	1.8 ± 0.4	1.0 ± 0.4
Cisplatin (3.5 mg/kg)	1293 ± 86	28.1 ± 6.9	13.1 ± 2.1	2.2 ± 0.4	1.0 ± 0.1

There are no significant differences between control and treatment groups (Steel's test, $p < 0.05$).

Table S2. Ratios of all detected *N*-glycans (μM) on the final day/pre-administration

i. High mannose

Peak No.	*m/z*	Presumptive composition	Cont.-	Cont.-	A-1	B-1	B-2	B-4	C-1	C-2	C-3	C-4
1	1362.481	(Hex)2 + (Man)3 (GlcNAc)2	0.85	0.82	1.90	1.02	1.37	0.85	1.07	0.90	0.93	0.73
2	1524.534	(Hex)3 + (Man)3 (GlcNAc)2	0.85	0.84	1.20	0.91	0.83	0.66	0.98	0.88	0.94	0.64
3	1686.587	(Hex)4 + (Man)3 (GlcNAc)2	0.91	0.83	0.92	0.68	0.72	0.58	1.07	0.86	1.01	0.65
4	1848.640	(Hex)5 + (Man)3 (GlcNAc)2	0.91	0.85	0.92	0.62	0.81	0.62	1.11	0.84	1.00	0.67
5	2010.692	(Hex)6 + (Man)3 (GlcNAc)2	0.89	0.88	0.86	0.60	0.74	0.58	1.06	0.81	0.92	0.65
6	2172.745	(Hex)7 + (Man)3 (GlcNAc)2	0.84	0.91	0.73	0.51	0.55	0.46	0.94	0.65	0.75	0.55

ii. Complex type, Hybrid type

Peak No.	*m/z*	Presumptive composition	Cont.-	Cont.-	A-1	B-1	B-2	B-4	C-1	C-2	C-3	C-4
7	1565.560	(Hex)2 (HexNAc)1 + (Man)3 (GlcNAc)2	N. A.	0.00	N. A.	N. A.	N. A.	N. A.	1.06	1.04	1.00	0.89
8	1708.619	(Hex)1 (HexNAc)1 (NeuAc)1 + (Man)3 (GlcNAc)2	1.09	0.92	1.98	1.74	1.67	0.93	1.17	1.00	1.00	0.90
9	1727.613	(Hex)3 (HexNAc)1 + (Man)3 (GlcNAc)2	0.90	0.79	2.83	1.22	1.09	0.72	1.11	1.00	1.04	0.88
10	1793.671	(HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	0.74	0.71	0.00	0.00	0.00	0.00	0.94	0.70	0.85	0.55

11	1809.666	(Hex)1 (HexNAc)3 + (Man)3 (GlcNAc)2	0.82	0.71	1.00	0.70	0.69	0.00	0.85	0.78	0.85	0.51
12	1870.672	(Hex)2 (HexNAc)1 (NeuAc)1 + (Man)3 (GlcNAc)2	0.96	0.92	1.93	1.59	1.32	0.86	1.13	0.92	1.02	0.77
13	1914.698	(Hex)2 (HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	0.97	0.87	0.00	N. A.	0.00	0.00	0.88	0.60	0.83	0.53
14	1955.724	(Hex)1 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	0.76	0.81	0.77	0.48	0.58	0.46	1.01	0.66	0.75	0.58
15	1971.719	(Hex)2 (HexNAc)3 + (Man)3 (GlcNAc)2	0.66	N. A.	N. A.	N. A.	0.00	N. A.	0.00	N. A.	N. A.	0.00
16	2032.724	(Hex)3 (HexNAc)1 (NeuAc)1 + (Man)3 (GlcNAc)2	0.96	0.89	3.27	2.36	1.78	0.98	1.15	0.98	0.99	0.94
17	2073.751	(Hex)2 (HexNAc)2 (NeuAc)1 + (Man)3 (GlcNAc)2	0.87	0.81	3.01	1.69	1.95	0.84	1.14	1.11	0.96	1.07
18	2117.777	(Hex)2 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	0.88	0.89	1.16	0.76	0.66	0.52	1.07	0.75	0.82	0.60
19	2219.809	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man)3	0.90	0.87	0.90	0.67	0.64	0.52	0.88	0.65	0.87	0.49
20	2235.804	(Hex)3 (HexNAc)2 (NeuAc)1 + (Man)3 (GlcNAc)2	N. A.	0.90	4.47	2.59	1.49	0.75	1.12	0.89	N. A.	0.86
21	2260.835	(Hex)1 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man)3	0.82	0.83	0.86	0.58	0.00	0.59	1.08	0.65	0.95	0.55
22	2276.830	(Hex)2 (HexNAc)3 (NeuAc)1 + (Man)3 (GlcNAc)2	0.80	N. A.	0.00	0.00	0.60	N. A.	0.95	0.00	0.78	0.00
23	2279.830	(Hex)3 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	N. A.	N. A.	N. A.	N. A.	N. A.	N. A.	1.06	0.00	0.00	N. A.
24	2304.862	(Hex)1 (HexNAc)4 (Deoxyhexose)2 + (Man)3 (GlcNAc)2	N. A.	0.81	2.77	1.69	1.54	0.00	1.23	0.67	0.88	0.00
25	2321.841	(Hex)2 (HexNAc)1 (Deoxyhexose)1 (NeuAc)2 + (Man)3	N. A.	N. A.	N. A.	N. A.	N. A.	N. A.	1.05	0.80	0.91	0.73
26	2336.851	(Hex)3 (HexNAc)4 + (Man)3 (GlcNAc)2	0.88	0.92	2.17	1.67	N. A.	0.79	1.14	0.81	0.88	0.71
27	2378.862	(Hex)2 (HexNAc)2 (NeuAc)2 + (Man)3 (GlcNAc)2	0.95	0.95	2.33	1.73	1.72	0.85	1.04	0.69	0.90	0.56
28	2422.888	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man)3	0.88	0.89	1.13	0.57	0.66	0.47	1.15	0.99	0.98	0.95

29	2438.883	(Hex)3 (HexNAc)3 (NeuAc)1 + (Man)3 (GlcNAc)2	0.85	0.84	3.96	2.34	1.99	0.84	N. A.	N. A.	N. A.	N. A.
30	2482.909	(Hex)3 (HexNAc)4 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	0.87	0.79	1.65	0.00	0.00	0.83	0.90	0.60	0.86	0.49
31	2524.920	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)2 + (Man)3	0.75	0.99	1.29	0.80	0.51	0.56	1.07	0.63	1.06	0.00
32	2727.999	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2 + (Man)3	1.00	0.91	0.99	0.68	0.59	0.51	1.09	0.64	1.12	0.55
33	2743.994	(Hex)3 (HexNAc)3 (NeuAc)2 + (Man)3 (GlcNAc)2	0.95	0.90	2.74	1.45	1.64	0.89	1.13	0.88	1.01	0.75
34	2788.020	(Hex)3 (HexNAc)4 (Deoxyhexose)1 (NeuAc)1 + (Man)3	0.78	0.91	0.00	0.00	0.00	0.00	N. A.	N. A.	N. A.	N. A.
35	2804.015	(Hex)4 (HexNAc)4 (NeuAc)1 + (Man)3 (GlcNAc)2	N. A.	0.00	2.4/N.	2.84	1.4/N.	0.00	1.25	0.90	1.05	0.80
36	2890.052	(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2 + (Man)3	N. A.	N. A.	N. A.	N. A.	N. A.	N. A.	N. A.	N. A.	N. A.	N. A.
37	3049.105	(Hex)3 (HexNAc)3 (NeuAc)3 + (Man)3 (GlcNAc)2	1.05	1.08	1.63	1.08	1.09	0.80	1.04	0.63	0.85	0.46
38	3109.126	(Hex)4 (HexNAc)4 (NeuAc)2 + (Man)3 (GlcNAc)2	0.99	1.01	3.81	2.21	2.08	1.16	1.17	0.71	0.96	0.58
39	3195.163	(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)3 + (Man)3	N. A.	1.06	N. A.	N. A.	N. A.	0.00	N. A.	N. A.	N. A.	N. A.
40	3414.237	(Hex)4 (HexNAc)4 (NeuAc)3 + (Man)3 (GlcNAc)2	N. A.	1.21	2.84	1.76	2.03	N. A.	1.14	0.61	0.89	0.00

N. A. not available ; N.D.: not detectable

Table S3. Ratios of increased *N*-glycans (μM) on the final day/pre-administration in diclofenac-treated group

ID	Kidney damage score	No. 8	No. 9	No. 12	No. 16	No. 17	No. 20	No. 24	No. 26	No. 27	No. 29	No. 33	No. 35	No. 38	No. 40
B-4	1	0.9	0.7	0.9	1.0	0.8	0.8	0.0	0.8	0.8	0.8	0.9	0.0	1.2	N. D./N. D.
B-2	2	1.7	1.1	1.3	1.8	1.9	1.5	1.5	1.1/N. D.	1.7	2.0	1.6	1.4/N. D.	2.1	2.0
A-1	4	2.0	2.8	1.9	3.3	3.0	4.5	2.8	2.2	2.3	4.0	2.7	2.4/N. D.	3.8	2.8
B-1	5	1.7	1.2	1.6	2.4	1.7	2.6	1.7	1.7	1.7	2.3	1.4	2.8	2.2	1.8

N.D.: not detectable

Table S4. Expression levels of glycans (No. 24, 33, and 38) and UA level in chickens A-1 (a) and B-4 (b) in which UA levels exceeded the normal range (2.5 – 8.1 mg/dL) after several days. In the case of glycans, the levels are shown as the ratio to the plasma mixture of healthy chickens.

a: Chicken A-1

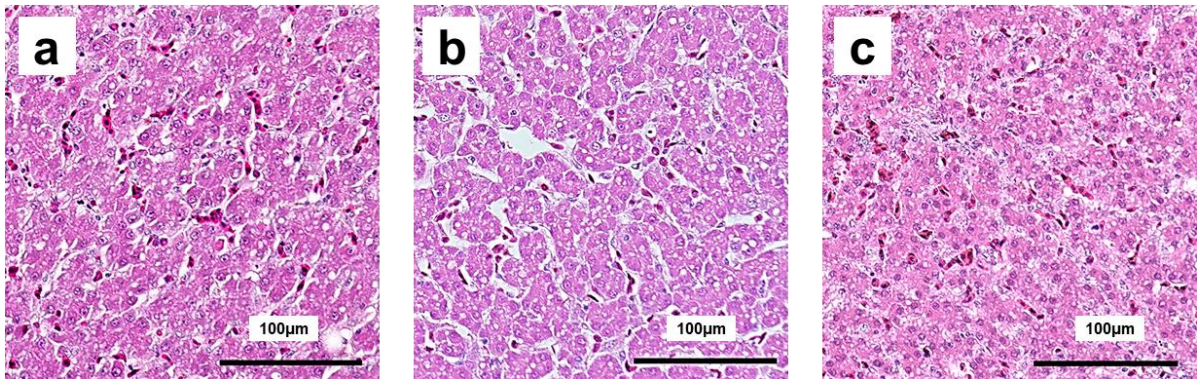
	Pre	24 h	48 h	72 h	96 h
Glycan No. 24	0.8	1.1	1.2	2.2	2.2
Glycan No.33	0.8	1.3	1.2	2.1	2.2
Glycan No.38	0.6	1.3	1.1	3.0	2.4
UA (mg/dL)	3.2	4.0	3.4	> 8.1	> 8.1

b: Chicken B-4

	Pre	24 h	48 h	72 h	96 h
Glycan No. 24	0.9	0.9	1.0	1.2	0.0
Glycan No.33	1.1	1.1	1.3	1.8	1.0
Glycan No.38	0.7	0.9	1.1	1.7	0.8
UA (mg/dL)	3.8	3.0	6.3	> 8.1	5.9

Fig. S1. Histopathological features of the liver in diclofenac- or cisplatin-treated and control chickens.

Chickens treated with diclofenac (a) or cisplatin (b) showed mild hyperemia compared with controls (c).



Note 5-1

Hematological tests in diclofenac-treated chickens

Objective

Experiment related to the chapter 5 and chapter 6 those mentioned about discovery of renal biomarkers in birds. In the diclofenac-treated group, hematological tests were carried out to clarify the effects of the drug on the hematology.

Materials and Methods

Experimental design

Male white leghorn chickens (*Gallus gallus domesticus*) (*n* = 11, 10 weeks, body weight: 1.2 – 1.4 kg) were purchased from Hokudo Co., Ltd. (Tokyo, Japan) and were housed under conditions of constant temperature (20°C ± 2°C) and humidity (40% ± 10%), with a 12:12-hour light:dark cycle and given food and water *ad libitum*. They were acclimatized in the environment for 1 week before commencement of the experiment. Pre-administration plasma was collected 1 week after arrival. Administration was started after a further 1 week. They were assigned randomly to one of three groups and received injection of either 20% dimethyl sulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan) diluted in saline or diclofenac sodium (Tokyo Chemical Industry Co., Tokyo, Japan) diluted in 20% DMSO into the pectoral muscle once daily in the morning for 4 consecutive days. The following groups were considered: control group (*n* = 3, ID: Cont.-1, 2, 3): injected 20% DMSO; diclofenac sodium group A (1.5 mg/kg body weight) (*n* = 4, ID: A-1, 2, 3, 4); diclofenac sodium group B (2.0 mg/kg body weight) (*n* = 4, ID: B-1,

2, 3, 4). These dose were decided based on the results of preliminary experiments and previous reports.

Blood collection and Hematological tests

Samples of 200 μL of whole blood were collected from the basilic vein before dissection using a 24 G needle without heparin and immediately moved into tubes containing ethylenediaminetetraacetic acid (EDTA). Hematocrit (Ht or HCT, also called packed cell volume) was measured using a microhematocrit centrifuge (Kubota 3220; Kubota Corporation, Tokyo, Japan) at 15000 × *g* for 5 minutes. Red blood cells (RBC) were counted using a Neubauer improved cell counting chamber after staining of blood with Natt and Herrick solution. White blood cell (WBC) count was calculated from the numbers of heterophils and eosinophils and the percentage of each white blood cell type. Heterophils and eosinophils were counted using a Neubauer improved cell counting chamber after staining with Randolph solution. Blood cells were counted within 12 hours of blood collection. The percentages of each white blood cell type were counted on blood smears stained with Giemsa solution.

Results

The results of hematological tests are shown in Table 1. One chicken (ID: B-1) died on the 3rd day and we could not collect post-injection blood with EDTA from this chicken. Hematocrit and total RBC number were not altered by the injection, and all individuals showed background levels. Total WBC increased in B-2 and A-1, which showed renal dysfunction.

Discussion

Hematological tests indicated that total WBC increased in A-1 and B-2, which showed renal dysfunction. The circulating concentrations of heterophils are increased by stresses and toxicants, and removal of 30% of the blood. In addition, heterophils are present in the urine of raptors with renal damage. Although the urine of chickens could not be collected, it is possible that heterophil numbers increased within the bodies of chickens with renal lesions. The same volume of blood was collected from each chicken, and heterophils did not increase in other chickens. Therefore, the increases in heterophil numbers in these two chickens would have been due to the toxicity of diclofenac and the renal lesions, rather than blood loss. Histopathological analysis demonstrated the infiltration of heterophils in the kidney, which seemed to be associated with their increase in the blood.

Table 1. RBC (red blood cells), Ht (hematocrit), MCV (mean corpuscular volume), WBC (white blood cells), and the ratio of each white blood cell type in diclofenac-treated and control chickens.

ID	RBC ($\times 10^6/mm^3$)	Ht (%)	MCV (fL)	WBC ($\times 10^3/mm^3$)	Eosinophils (%)	Heterophils (%)	Basophils (%)	Monocytes (%)	Lymphocytes (%)
Cont.-1	3.9	28.2	72.7	33.0	1.5	21.2	1.5	15.2	60.6
Cont.-2	2.8	22.0	78.9	14.5	2.7	27.0	2.7	10.8	56.8
Cont.-3	NA	NA	NA	NA	NA	NA	NA	NA	NA
A-1	3.1	22.8	73.3	56.1	0.9	79.3	1.8	4.5	13.5
A-2	3.1	24.0	78.2	38.1	0.0	18.2	1.8	5.5	74.5
A-3	3.7	25.2	68.3	19.4	2.6	26.9	0.0	10.3	60.3
A-4	3.1	25.2	82.4	28.5	0.0	20.0	0.0	7.3	72.7
B-1	NA	NA	NA	NA	NA	NA	NA	NA	NA
B-2	3.6	25.5	71.4	45.2	0.0	38.2	0.0	9.0	52.8
B-3	3.3	23.0	69.9	22.0	1.9	28.3	0.0	9.4	60.4
B-4	2.9	25.0	85.0	33.0	1.3	25.3	2.5	6.3	64.6

Note 5-2

Glycan expressions in chickens in chapter 5

Background and Objective

Expression levels of each glycan were showed by the ratio with the plasma mixture of healthy chickens including chickens in chapter 5. It is because for the comparison with another result that measured different time, the results shown with the ratio with mixture is available. However, expression levels were various among glycans. Therefore, the glycan expression levels are shown as a supporting data (Table 1).

Table 1-(1). *N*-glycans detected in chickens and expression level of each glycan (μM) in control chickens (Cont.-1 and -2) and diclofenac-treated chickens (A-1, B-1, B-2 and B-4).

i. High mannose type

Peak No.	*m/z*	Pre-administration						Post-administration					
		Cont.-1	Cont.-2	A-1	B-1	B-2	B-4	Cont.-1	Cont.-2	A-1	B-1	B-2	B-4
1	1362.481	3.244	2.737	2.884	2.953	2.528	3.318	2.713	2.177	5.825	3.022	3.622	2.777
2	1524.534	1.549	1.492	1.230	1.459	1.429	1.668	1.294	1.242	1.492	1.312	1.162	1.070
3	1686.587	1.157	1.137	1.020	1.236	1.147	1.335	1.049	0.932	0.934	0.827	0.804	0.751
4	1848.640	2.716	2.678	2.254	3.173	2.521	2.976	2.457	2.237	2.065	1.878	2.006	1.770
5	2010.692	8.383	8.059	6.651	9.802	7.774	9.652	7.403	7.025	5.610	5.537	5.513	5.326
6	2172.745	20.599	18.140	14.765	21.879	20.346	22.812	17.203	16.351	10.436	10.629	10.813	9.846

ii. Complex type, Hybrid type

7	1565.560		0.310							0.847	0.557	0.460	
8	1708.619	0.980	1.031	1.029	1.108	0.875	1.083	1.082	0.939	2.166	2.022	1.547	0.996

9	1727.613	0.707	0.621	0.557	0.707	0.645	0.761	0.631	0.473	1.721	0.879	0.708	0.525
10	1793.671	0.875	0.826	0.682	0.838	0.820	0.802	0.638	0.572				
11	1809.666	0.745	0.943	0.741	1.045	0.878	0.991	0.592	0.633	0.740	0.693	0.564	
12	1870.672	1.542	1.569	1.557	1.546	1.458	1.679	1.478	1.422	3.187	2.566	1.987	1.412
13	1914.698	0.814	0.637	0.656		0.826	0.876	0.787	0.564		0.351		
14	1955.724	7.287	6.999	6.161	7.762	7.619	7.705	5.462	5.644	4.669	3.637	4.298	3.405
15	1971.719	1.432				2.041		1.103	0.402		1.183		
16	2032.724	2.705	2.599	2.484	2.599	2.142	2.671	2.581	2.305	8.321	6.241	3.873	2.618
17	2073.751	50.400	47.876	44.005	56.105	40.512	55.123	43.978	38.750	130.241	94.234	78.043	46.675
18	2117.777	9.435	11.723	9.278	12.446	12.037	14.023	8.297	10.432	10.808	9.479	7.985	7.259
19	2219.809	2.336	2.562	1.890	2.614	2.229	3.096	2.096	2.216	1.698	1.699	1.376	1.557
20	2235.804		0.568	0.490	0.596	0.523	0.610	0.491	0.504	2.395	1.638	0.809	0.446
21	2260.835	2.060	2.381	1.654	1.860	1.898	1.793	1.798	2.068	1.506	1.320		1.287
22	2276.830	1.392		1.302	1.800	2.830		1.098	1.147			1.651	
23	2279.830												
24	2304.862		1.397	1.257	1.519	1.319	1.322	1.422	1.234	2.492	2.180	1.727	
25	2321.841												

26	2336.851	1.701	1.487	1.304	1.592		1.476	1.505	1.375	2.799	2.648	2.006	1.173
27	2378.862	72.111	64.353	64.925	70.042	59.130	66.265	68.961	61.247	147.506	119.266	99.568	56.514
28	2422.888	13.767	15.440	10.840	15.555	14.188	15.323	12.106	13.713	12.207	8.958	9.421	7.355
29	2438.883	4.822	4.513	3.423	5.289	3.978	5.280	4.083	3.790	13.447	12.317	7.875	4.451
30	2482.909	1.767	1.707	1.481	1.893	1.661	1.473	1.535	1.353	2.421			1.231
31	2524.920	1.593	2.395	1.134	1.211	1.028	3.065	1.223	2.365	1.428	0.987	0.575	1.755
32	2727.999	1.788	1.964	1.086	1.722	1.471	1.481	1.789	1.793	1.074	1.212	0.921	0.817
33	2743.994	6.501	7.370	5.951	8.341	6.011	8.017	6.164	6.616	16.106	12.026	9.791	7.120
34	2788.020	1.245	1.139	0.973	1.054	1.157	1.222	1.006	1.049				
35	2804.015		0.718		0.862		0.786			2.419	2.278	1.440	
36	2890.052		0.349				0.444		0.332				
37	3049.105	8.423	9.828	8.839	8.575	8.168	9.421	8.789	10.565	14.015	9.222	8.819	7.664
38	3109.126	0.802	0.761	0.611	0.850	0.656	0.656	0.796	0.766	2.074	1.768	1.268	0.748
39	3195.163		0.318				0.402		0.336				
40	3414.237		0.278	0.245	0.314	0.240		0.319	0.317	0.513	0.478	0.384	

Table 1-(2). *N*-glycans detected in chickens and expression level of each glycan (μM) in cisplatin-treated chickens (C-1, -2, -3, -4).

i. High mannose type

Peak No.	*m/z*	Pre-administration				Post-administration			
		C-1	C-2	C-3	C-4	C-1	C-2	C-3	C-4
1	1362.481	2.571	3.067	3.316	3.629	2.883	2.593	2.935	2.171
2	1524.534	0.861	0.870	0.940	1.155	0.834	0.713	0.859	0.579
3	1686.587	0.876	0.909	0.886	0.851	0.964	0.738	0.901	0.447
4	1848.640	2.705	2.517	2.553	2.220	3.047	2.041	2.551	1.332
5	2010.692	8.886	8.403	9.350	7.289	9.445	6.692	8.519	4.497
6	2172.745	22.382	19.688	24.411	19.099	20.986	13.110	18.554	10.738

ii. Complex type, Hybrid type

7	1565.560	0.250	0.234	0.234	0.315	0.276	0.253	0.235	0.256
8	1708.619	0.697	0.779	0.757	0.842	0.880	0.780	0.760	0.723
9	1727.613	0.574	0.607	0.550	0.636	0.674	0.607	0.583	0.511
10	1793.671	0.821	0.977	0.773	1.591	0.756	0.579	0.609	0.716

11	1809.666	1.010	0.756	0.802	1.511	0.798	0.502	0.618	0.564
12	1870.672	1.329	1.404	1.278	1.353	1.586	1.238	1.314	0.910
13	1914.698	0.659	0.557	0.733	0.758	0.552	0.244	0.567	0.289
14	1955.724	7.264	6.934	7.476	10.455	7.309	4.079	5.274	5.422
15	1971.719	1.838			2.393				
16	2032.724	2.044	2.338	2.187	1.958	2.440	2.264	2.147	1.791
17	2073.751	48.320	50.948	54.958	50.236	55.060	56.138	52.598	53.487
18	2117.777	14.851	10.944	19.587	14.816	16.018	7.850	15.852	8.246
19	2219.809	2.100	1.885	2.306	1.810	1.799	1.080	1.941	0.685
20	2235.804	0.344	0.378		0.325	0.395	0.328	0.313	0.271
21	2260.835	1.588	1.873	1.567	2.272	1.704	1.275	1.492	1.330
22	2276.830	1.099	0.960	1.121	1.083	1.027		0.798	
23	2279.830	0.426	0.324	0.441		0.461			
24	2304.862	0.975	1.041	1.230	1.004	1.134	0.787	1.118	
26	2336.851	1.134	1.129	1.366	1.102	1.204	0.859	1.220	0.740
27	2378.862	63.367	72.789	86.116	62.972	71.096	60.043	76.582	46.951
28	2422.888	14.011	13.808	16.368	13.797	14.573	9.322	14.621	7.448

29	2438.883	4.824	5.335	5.618	4.865	5.598	5.286	5.513	4.616
30	2482.909								
31	2524.920	1.167	1.255	1.606	0.749	1.030	0.687	1.353	0.285
32	2727.999	1.429	1.679	1.276	1.057	1.562	1.052	1.434	0.565
33	2743.994	7.936	9.050	10.081	8.201	9.010	7.972	10.227	6.110
35	2804.015	0.779	0.850	0.854	0.705	0.987	0.756	0.897	0.550
36	2890.052								
37	3049.105	7.423	9.708	11.890	7.172	7.738	6.280	10.175	3.516
38	3109.126	0.659	0.816	0.828	0.534	0.776	0.577	0.790	0.304
40	3414.237	0.133	0.163	0.176	0.119	0.149	0.107	0.158	

Note 5-3

Glycan expressions in Pb exposed chicken

Background and Objective

Chapter 5 showed that glycan expression reflects the kidney injury caused by diclofenac. However, in the cisplatin group, glycan levels did not change significantly according to kidney injury, although the renal lesions were severe and uric acid concentrations were high.

The mechanisms of metabolism of diclofenac and cisplatin are different. Therefore, it is possible that glycans reflect the limited pathways of kidney injury, and glycan expression profiles change due to AIN but do not reflect ATN. Although further studies, including investigation of species differences, are needed, glycans have the potential to be useful biomarkers for AIN in avian species. The glycan expression profile may reflect some types of kidney injury, and these molecules have the potential for use as biomarkers for the evaluation of functional disorders in birds.

In the current study, glycan expressions were measured in chickens exposed to Pb in Zambia. Pb induces activation of transcription nuclear factor kappa B, activation of the intrarenal renin-angiotensin system, and attraction of macrophages, which generates an inflammatory process in the renal interstitium that may be involved in the development of tubulointerstitial damage and high blood pressure (Bravo et al., 2007; Sabath and Robles-Osorio, 2012).

Materials and Methods

In vivo *experiment using the broiler chicken (*Gallus gallus domesticus*)*

This *in vivo* experiment was carried out in Zambia for the SATREPS (Science and Technology Research Partnership for Sustainable Development) project by JST/JICA, and they provided plasma specimens for my research. The animal experiment was performed under the supervision and with the approval of the Institutional Animal Care and Use Committee of Hokkaido University, Japan (approval number: 14-0119).

Male broiler chickens (n = 16, body weight: 2.0−3.5 kg) were provided by local farms in Zambia. They were acclimatized to the laboratory environment with a 12: 12-hour light: dark cycle and given food and water *ad libitum* for one week before the commencement of the experiment at the School of Veterinary Medicine, University of Zambia, Lusaka, Zambia. After acclimation, they were randomly assigned to two groups and injected with either saline or Pb (II) acetate trihydrate (Wako Pure Chemicals Industries, Osaka, Japan) in the pectoral muscle. The following groups were considered: a control group injected with saline water, and a high-dose group given Pb (II) acetate trihydrate (10 mg/kg body weight) (n = 4 in each group). 24 hours after the intramuscular injection, the chickens were euthanized, and kidney and blood samples were collected. Kidney specimens were cut into small pieces and stored in 10% neutral buffered formalin for histopathological analysis. As for the blood samples, plasma samples were collected after centrifugation, then preserved at -20 °C. Samples were transported to the Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan for analysis.

Histopathological Analysis

Paraffin-embedded kidney sections were stained with periodic acid-Schiff, and liver, heart, and lung sections were stained with hematoxylin and eosin.

Glycoblotting-based plasma glycomics

An analysis of glycans followed the protocol described in chapter 5.

Results and Discussion

Histopathological tests did not show renal failure in either control or Pb exposure groups (Fig. 1).

Results of glycan expression showed that there were many individual differences even within a treatment group, and there might be no candidate biomarker linked to Pb exposure (Table 1 - [1] and [2]). Expression levels of each glycan are shown as a ratio with the plasma mixture (plasma samples were pooled) of healthy chickens, including the chickens in chapter 5 (Table 1) or the expression level itself (Table 2). The backgrounds (including age and breeding place) of these chickens varied. Furthermore, acute Pb exposure was only 24 hours prior to sampling. In this study, intramuscular injection was used, which might take a little more time to cause renal failure. Histopathological analysis also showed that the kidneys were not damaged. There was a possibility that kidney function was still fine and glycan levels did not react. However, individual chickens showed different expression levels of glycans, which indicated that glycan expressions vary in the wild, even in healthy birds of the same species.

Fig. 1.

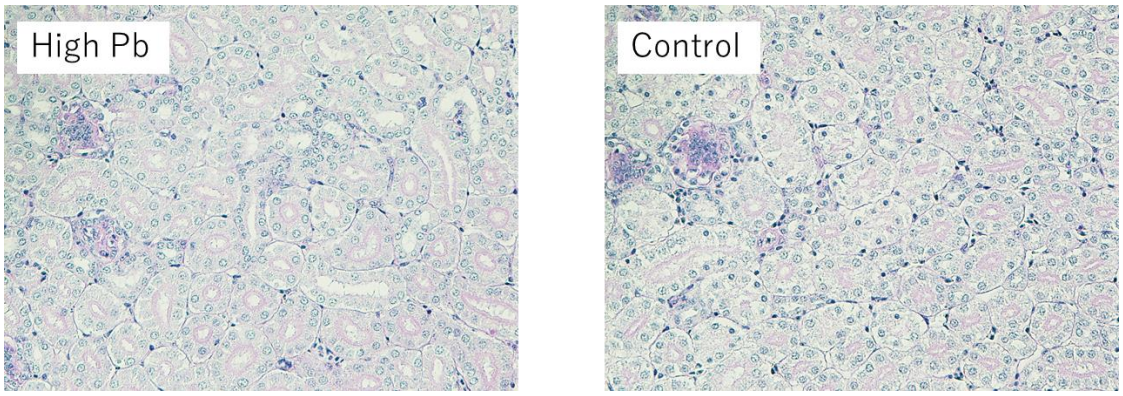


Table 1-(1). Ratios of all detected *N*-glycans (μM) in chickens from control group. Expression levels of each glycan were showed by the ratio with the plasma mixture of healthy chickens including chickens in chapter 4.

a) High-mannose type

Peak No.	*m/z*	Presumptive composition	Control_1	Control_2	Control_3	Control_4	Average of Controls	SD of Controls
1	1362.481	(Hex)2 + (Man)3 (GlcNAc)2	1.09	1.08	0.97	1.34	1.12	0.14
2	1524.534	(Hex)3 + (Man)3 (GlcNAc)2	1.17	0.98	0.83	0.98	0.99	0.12
3	1686.587	(Hex)4 + (Man)3 (GlcNAc)2	1.22	1.27	0.84	1.00	1.08	0.17
4	1848.640	(Hex)5 + (Man)3 (GlcNAc)2	1.02	1.16	0.82	1.34	1.09	0.19
5	2010.692	(Hex)6 + (Man)3 (GlcNAc)2	0.81	1.11	0.85	1.78	1.14	0.39
6	2172.745	(Hex)7 + (Man)3 (GlcNAc)2	0.65	1.03	0.89	2.57	1.29	0.75

b) Complex type, Hybrid type

7	1565.560	(Hex)2 (HexNAc)1 + (Man)3 (GlcNAc)2	0.00	1.01	0.00	1.00	0.50	0.50
8	1708.619	(Hex)1 (HexNAc)1 (NeuAc)1 + (Man)3 (GlcNAc)2	0.92	1.02	0.93	1.66	1.13	0.31
9	1727.613	(Hex)3 (HexNAc)1 + (Man)3 (GlcNAc)2	1.00	1.32	0.66	1.06	1.01	0.23
10	1793.671	(HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	1.17	0.96	1.86	0.00	1.00	0.67

11	1809.666	(Hex)1 (HexNAc)3 + (Man)3 (GlcNAc)2	1.15	1.09	1.12	1.21	1.14	0.05
12	1870.672	(Hex)2 (HexNAc)1 (NeuAc)1 + (Man)3 (GlcNAc)2	0.99	1.17	0.99	1.41	1.14	0.17
13	1914.698	(Hex)2 (HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	1.61	1.08	0.80	1.49	1.25	0.32
14	1955.724	(Hex)1 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	0.96	0.88	1.21	0.73	0.95	0.18
15	1971.719	(Hex)2 (HexNAc)3 + (Man)3 (GlcNAc)2	1.35	1.29	0.61	0.91	1.04	0.30
16	2032.724	(Hex)3 (HexNAc)1 (NeuAc)1 + (Man)3 (GlcNAc)2	0.94	1.16	0.90	1.49	1.12	0.24
17	2073.751	(Hex)2 (HexNAc)2 (NeuAc)1 + (Man)3 (GlcNAc)2	0.96	1.07	0.73	1.65	1.10	0.34
18	2117.777	(Hex)2 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	1.15	1.04	0.72	1.36	1.07	0.23
19	2219.809	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNAc)2	1.50	1.58	0.96	1.40	1.36	0.24
20	2235.804	(Hex)3 (HexNAc)2 (NeuAc)1 + (Man)3 (GlcNAc)2	0.86	0.00	0.69	1.64	0.80	0.58
21	2260.835	(Hex)1 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNAc)2	0.99	0.91	1.09	0.00	0.75	0.44
22	2276.830	(Hex)2 (HexNAc)3 (NeuAc)1 + (Man)3 (GlcNAc)2	1.83	2.03	1.62	0.72	1.55	0.50
23	2279.830	(Hex)3 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	0.00	0.00	0.00	0.00	0.00	0.00
24	2304.862	(Hex)1 (HexNAc)4 (Deoxyhexose)2 + (Man)3 (GlcNAc)2	1.07	0.85	0.91	2.02	1.21	0.47
25	2321.841	(Hex)2 (HexNAc)1 (Deoxyhexose)1 (NeuAc)2 + (Man)3 (GlcNAc)2	0.91	1.77	0.00	2.66	1.34	0.99
26	2336.851	(Hex)3 (HexNAc)4 + (Man)3 (GlcNAc)2	0.93	0.81	0.92	2.23	1.22	0.58
27	2378.862	(Hex)2 (HexNAc)2 (NeuAc)2 + (Man)3 (GlcNAc)2	1.04	0.83	0.93	1.87	1.17	0.41
28	2422.888	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNAc)2	1.12	1.06	1.01	1.14	1.08	0.05

29	2438.883	(Hex)3 (HexNAc)3 (NeuAc)1 + (Man)3 (GlcNAc)2	0.96	1.38	1.10	1.83	1.32	0.33
30	2482.909	(Hex)3 (HexNAc)4 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	0.98	0.00	0.00	3.98	1.24	1.63
31	2524.920	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)2 + (Man)3 (GlcNAc)2	1.68	1.91	0.92	2.93	1.86	0.72
32	2727.999	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2 + (Man)3 (GlcNAc)2	0.95	1.34	1.07	1.41	1.19	0.19
33	2743.994	(Hex)3 (HexNAc)3 (NeuAc)2 + (Man)3 (GlcNAc)2	1.12	1.27	1.30	1.82	1.38	0.27
34	2788.020	(Hex)3 (HexNAc)4 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNAc)2	0.00	0.91	1.05	3.65	1.40	1.36
35	2804.015	(Hex)4 (HexNAc)4 (NeuAc)1 + (Man)3 (GlcNAc)2	1.12	1.88	1.69	2.14	1.71	0.38
36	2890.052	(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2 + (Man)3 (GlcNAc)2	0.00	0.00	0.00	0.00	0.00	0.00
37	3049.105	(Hex)3 (HexNAc)3 (NeuAc)3 + (Man)3 (GlcNAc)2	1.12	0.93	1.17	2.07	1.32	0.44
38	3109.126	(Hex)4 (HexNAc)4 (NeuAc)2 + (Man)3 (GlcNAc)2	1.29	1.71	1.98	2.61	1.90	0.48
39	3195.163	(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)3 + (Man)3 (GlcNAc)2	1.35	1.64	0.00	3.97	1.74	1.43
40	3414.237	(Hex)4 (HexNAc)4 (NeuAc)3 + (Man)3 (GlcNAc)2	1.30	1.44	3.59	2.69	2.26	0.94

Table 1-(2). Ratios of all detected *N*-glycans (μM) in chickens from high Pb group. Expression levels of each glycan were showed by the ratio with the plasma mixture of healthy chickens including chickens in chapter 4.

a) High-mannose

Peak No.	*m/z*	Presumptive composition	High_1	High_2	High_3	High_4	Average of high dose group	SD of high dose group	Average of High/Control
1	1362.481	(Hex)2 + (Man)3 (GlcNAc)2	0.90	0.98	1.17	0.64	0.92	0.19	0.82
2	1524.534	(Hex)3 + (Man)3 (GlcNAc)2	0.83	0.92	1.02	0.60	0.84	0.15	0.85
3	1686.587	(Hex)4 + (Man)3 (GlcNAc)2	0.92	0.96	1.14	0.63	0.91	0.18	0.84
4	1848.640	(Hex)5 + (Man)3 (GlcNAc)2	1.45	1.08	1.10	0.55	1.05	0.32	0.97
5	2010.692	(Hex)6 + (Man)3 (GlcNAc)2	2.18	1.38	0.89	0.45	1.22	0.64	1.08
6	2172.745	(Hex)7 + (Man)3 (GlcNAc)2	2.97	1.90	0.90	0.34	1.53	1.00	1.19
b) Complex type,									
7	1565.560	(Hex)2 (HexNAc)1 + (Man)3 (GlcNAc)2	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8	1708.619	(Hex)1 (HexNAc)1 (NeuAc)1 + (Man)3 (GlcNAc)2	0.83	1.02	1.02	1.06	0.98	0.09	0.87
9	1727.613	(Hex)3 (HexNAc)1 + (Man)3 (GlcNAc)2	0.75	0.82	0.90	0.65	0.78	0.09	0.77
10	1793.671	(HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	0.00	0.00	1.17	0.00	0.29	0.51	0.29

11	1809.666	(Hex)1 (HexNAc)3 + (Man)3 (GlcNAc)2	1.38	0.90	1.48	0.00	0.94	0.59	0.82
12	1870.672	(Hex)2 (HexNAc)1 (NeuAc)1 + (Man)3 (GlcNAc)2	0.88	0.98	0.98	1.05	0.97	0.06	0.85
13	1914.698	(Hex)2 (HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	1.70	1.24	1.23	0.65	1.21	0.37	0.97
14	1955.724	(Hex)1 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	0.63	0.69	1.05	0.46	0.70	0.22	0.74
15	1971.719	(Hex)2 (HexNAc)3 + (Man)3 (GlcNAc)2	3.33	0.95	1.25	0.00	1.38	1.22	1.33
16	2032.724	(Hex)3 (HexNAc)1 (NeuAc)1 + (Man)3 (GlcNAc)2	0.80	0.86	0.81	0.92	0.85	0.05	0.76
17	2073.751	(Hex)2 (HexNAc)2 (NeuAc)1 + (Man)3 (GlcNAc)2	0.75	0.87	1.00	0.66	0.82	0.13	0.74
18	2117.777	(Hex)2 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	1.65	1.27	1.18	0.49	1.15	0.42	1.08
19	2219.809	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man)3	1.25	1.26	1.29	0.75	1.14	0.23	0.84
20	2235.804	(Hex)3 (HexNAc)2 (NeuAc)1 + (Man)3 (GlcNAc)2	1.27	0.88	0.97	0.00	0.78	0.47	0.98
21	2260.835	(Hex)1 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man)3	0.00	0.00	0.00	0.00	0.00	0.00	0.00
22	2276.830	(Hex)2 (HexNAc)3 (NeuAc)1 + (Man)3 (GlcNAc)2	1.58	1.44	1.34	1.26	1.40	0.12	0.91
23	2279.830	(Hex)3 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	2.43	1.14	0.00	0.00	0.89	1.00	N. A.
24	2304.862	(Hex)1 (HexNAc)4 (Deoxyhexose)2 + (Man)3 (GlcNAc)2	0.00	0.86	0.00	0.91	0.44	0.44	0.36
25	2321.841	(Hex)2 (HexNAc)1 (Deoxyhexose)1 (NeuAc)2 + (Man)3	1.85	2.32	0.00	0.00	1.04	1.06	0.78
26	2336.851	(Hex)3 (HexNAc)4 + (Man)3 (GlcNAc)2	3.04	0.93	0.82	0.77	1.39	0.95	1.13
27	2378.862	(Hex)2 (HexNAc)2 (NeuAc)2 + (Man)3 (GlcNAc)2	0.86	0.89	0.91	1.03	0.92	0.06	0.79
28	2422.888	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man)3	0.71	1.22	0.97	0.50	0.85	0.27	0.78

29	2438.883	(Hex)3 (HexNAc)3 (NeuAc)1 + (Man)3 (GlcNAc)2	0.88	0.99	0.92	0.66	0.86	0.12	0.65
30	2482.909	(Hex)3 (HexNAc)4 (Deoxyhexose)1 + (Man)3 (GlcNAc)2	6.42	1.19	0.88	0.56	2.26	2.41	1.83
31	2524.920	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)2 + (Man)3	2.36	1.02	1.50	1.58	1.62	0.48	0.87
32	2727.999	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2 + (Man)3	0.54	0.00	0.68	0.50	0.43	0.26	0.36
33	2743.994	(Hex)3 (HexNAc)3 (NeuAc)2 + (Man)3 (GlcNAc)2	0.92	1.10	0.94	0.77	0.93	0.12	0.68
34	2788.020	(Hex)3 (HexNAc)4 (Deoxyhexose)1 (NeuAc)1 + (Man)3	4.54	1.77	0.00	0.00	1.58	1.86	1.12
35	2804.015	(Hex)4 (HexNAc)4 (NeuAc)1 + (Man)3 (GlcNAc)2	1.49	1.31	1.06	0.83	1.17	0.25	0.69
36	2890.052	(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2 + (Man)3	0.00	0.00	0.00	0.00	0.00	0.00	N. A.
37	3049.105	(Hex)3 (HexNAc)3 (NeuAc)3 + (Man)3 (GlcNAc)2	0.82	0.83	0.46	1.05	0.79	0.21	0.60
38	3109.126	(Hex)4 (HexNAc)4 (NeuAc)2 + (Man)3 (GlcNAc)2	0.84	1.16	0.68	0.99	0.92	0.18	0.48
39	3195.163	(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)3 + (Man)3	3.09	1.82	0.00	1.24	1.54	1.11	0.88
40	3414.237	(Hex)4 (HexNAc)4 (NeuAc)3 + (Man)3 (GlcNAc)2	0.00	0.89	0.00	2.11	0.75	0.86	0.33

Table 2. *N*-glycans detected in chickens and expression level of each glycan (μM)

a) High-mannose type

Peak No.	*m/z*	Control_1	Control_2	Control_3	Control_4	High_1	High_2	High_3	High_4
1	1362.481	3.411	3.387	2.976	4.301	2.752	3.028	3.693	1.834
2	1524.534	1.576	1.304	1.086	1.297	1.078	1.221	1.355	0.758
3	1686.587	1.247	1.309	0.843	1.020	0.929	0.971	1.168	0.619
4	1848.640	2.396	2.770	1.904	3.231	3.528	2.574	2.616	1.198
5	2010.692	5.878	8.304	6.198	13.818	17.057	10.575	6.523	2.888
6	2172.745	12.423	20.162	17.171	51.846	60.179	38.055	17.380	5.975

b) Complex type, Hybrid type

7	1565.560		0.353		0.353				
8	1708.619	0.979	1.101	0.987	1.863	0.871	1.104	1.094	1.144
9	1727.613	0.687	0.926	0.428	0.733	0.497	0.549	0.608	0.414
10	1793.671	0.695	0.561	1.134				0.693	
11	1809.666	0.952	0.891	0.925	1.007	1.168	0.714	1.263	

12	1870.672	1.700	2.039	1.695	2.505	1.499	1.675	1.687	1.809
13	1914.698	1.459	1.000	0.752	1.356	1.539	1.136	1.131	0.625
14	1955.724	4.660	4.286	5.972	3.508	2.979	3.292	5.118	2.111
15	1971.719	2.635	2.536	1.455	1.923	5.827	1.987	2.471	
16	2032.724	2.892	3.607	2.768	4.663	2.464	2.644	2.502	2.832
17	2073.751	49.498	55.363	37.876	84.887	38.850	45.382	51.705	34.358
18	2117.777	11.799	10.656	7.376	13.931	16.951	13.019	12.132	4.969
19	2219.809	3.635	3.854	2.289	3.390	3.030	3.055	3.118	1.755
20	2235.804	0.608		0.475	1.212	0.922	0.625	0.692	
21	2260.835	1.556	1.477	1.658					
22	2276.830	2.995	3.341	2.637	1.128	2.579	2.338	2.174	2.029
23	2279.830					1.401	0.633		
24	2304.862	1.490	1.299	1.355	2.316		1.309		1.350
25	2321.841	0.849	1.645		2.471	1.722	2.157		
26	2336.851	1.688	1.470	1.672	4.001	5.442	1.686	1.479	1.400
27	2378.862	71.960	57.789	65.049	127.559	60.415	62.370	63.405	71.587
28	2422.888	11.705	11.168	10.651	11.976	7.574	12.723	10.180	5.431

29	2438.883	5.198	7.482	5.981	9.909	4.782	5.384	5.002	3.588
30	2482.909	1.960			7.896	12.720	2.388	1.767	1.130
31	2524.920	3.715	4.207	2.084	6.395	5.176	2.307	3.320	3.507
32	2727.999	1.153	1.580	1.277	1.654	0.703		0.862	0.666
33	2743.994	8.335	9.467	9.624	13.493	6.890	8.188	7.001	5.738
34	2788.020		1.140	1.297	4.074	5.027	2.061		
35	2804.015	1.196	1.950	1.767	2.206	1.569	1.393	1.137	0.912
36	2890.052	0.515	0.634	0.384	1.241	1.295	0.564	0.417	0.389
37	3049.105	8.418	7.082	8.757	15.033	6.352	6.374	3.823	7.967
38	3109.126	1.159	1.513	1.739	2.258	0.788	1.056	0.656	0.914
39	3195.163	0.391	0.469		1.101	0.862	0.518		0.362
40	3414.237	0.413	0.446	0.965	0.747		0.313		0.607

References

Bravo, Y., Quiroz, Y., Ferrebuz, A., Vaziri, N.D., Rodríguez-Iturbe, B., 2007. Mycophenolate mofetil administration reduces renal inflammation, oxidative stress, and arterial pressure in rats with lead-induced hypertension. Am. J. Physiol. Physiol. 293, F616–F623.

Sabath, E., Robles-Osorio, M.L., 2012. Renal health and the environment: heavy metal nephrotoxicity. Nefrologia 32, 279–286.

CHAPTER 6

Discovery of novel renal biomarkers in a chicken model using transcriptome analysis

Abstract

Bird deaths are often caused by renal lesions induced by chemicals. The avian kidney has a renal portal system with significant blood flow that is sensitive to many chemicals. However, early biomarkers of kidney injury have not been identified in avians. The objective of this study is to identify novel renal biomarkers. Acute kidney injury (AKI) can be divided into acute interstitial nephritis (AIN) and acute tubular necrosis (ATN); a chicken model of kidney damage was created by injection of diclofenac or cisplatin, which causes either AIN or ATN, respectively. Microarray analysis was performed to profile gene expression patterns in chickens with nephritis. Gene ontology analysis suggested that in both AIN and ATN, genes related to responses to external stimuli showed expression changes. However, hierarchical clustering analyses suggested that AIN and ATN affected gene expression patterns differently, and the number of biomarkers relevant to renal damage was small. To identify early biomarkers of nephritis, we focused on genes that were induced at a variety of levels of renal damage; vanin-1 (VNN1) was found to be highly induced in early stages of renal damage. Results of quantitative real-time PCR also supported this finding. These results suggest that VNN1 could be a useful early biomarker of kidney injury in avian species.

Keywords: acute interstitial nephritis (AIN), acute tubular necrosis (ATN), bird, VNN1 (vanin-1), renal biomarker

Highlights

- Diclofenac induces acute interstitial nephritis (AIN), and cisplatin causes acute tubular necrosis (ATN) in chickens.
- Vanin-1 (VNN1) could be a useful early biomarker of kidney injury in avian species.

1. Introduction

Bird deaths are often caused by renal lesions induced by chemicals. For instance, diclofenac, a nonsteroidal anti-inflammatory drug (NSAID), has led to the deaths of many vultures in the Indian subcontinent after the accidental intake of diclofenac from consumption of cattle carcasses and the subsequent development of severe kidney injury (Green et al., 2004; Swan et al., 2006). Other therapeutic agents, such as cisplatin, also cause renal damage in birds. It is known that the threshold levels of these chemicals are very low in birds compared with other animals.

One of the reasons for this outcome is the structure of the avian kidney, which has a renal portal system that does not exist in mammalian species (Lierz, 2003). Because of this unique system, birds have significant blood flow into the kidney (Harr, 2002; Shideman et al., 1981) and are sensitive to many chemicals. Moreover, this unique system suggests that gene expression changes in birds, as a result of chemical exposure, might differ from those in mammalian species. Primary avian kidney cells have been shown to be much more susceptible than mammalian cells to both diclofenac and meloxicam (Naidoo and Swan, 2009).

In mammals, gene expression profiling in the kidney has been performed, and clinical information has been gathered (Yasuda et al., 2006). Moreover, kidney biomarkers have been identified in mammals. Blood urea nitrogen (BUN) and creatinine have been used as biomarkers of kidney injury at the hospital. Indeed, there are earlier biomarkers, such as neutrophil gelatinase-associated lipocalin (NGAL) (Cruz et al., 2010), cystatin C (Dharnidharka et al., 2002), kidney injury molecule-1 (KIM-1) (Han et al., 2002), and microRNA (Ichii et al., 2012), that have been used for mammalian kidney injury. However, avian species have no early biomarkers of renal damage. Uric acid (UA)

levels are generally used in birds. However, the levels of UA only change when the kidney is severely damaged, and high levels of UA typically link with death within a few days. Although phosphorus levels are useful in some birds, it is generally uncommon in avian species (Tully et al., 2009). Therefore, the discovery of novel biomarkers for kidney injury in birds is required.

The objective of the current study is to discover and identify novel renal biomarkers in avians. First, a chicken model of kidney damage was created by injection of cisplatin or diclofenac. Acute kidney injury (AKI) can be divided into acute interstitial nephritis (AIN) and acute tubular necrosis (ATN) (Hosohata, 2016). Diclofenac causes AIN, and cisplatin causes ATN. Therefore, these two chemicals were used to create different models of kidney damage in chickens.

Cisplatin is an inorganic, platinum-based chemotherapeutic agent. The kidney accumulates and retains more cisplatin than any other organ and its chief dose-limiting side effect is nephrotoxicity, (Arany and Safirstein, 2003). Cisplatin accumulates in all nephron segments but is preferentially taken up by highly susceptible proximal tubule cells (Mishra et al., 2004), and causes nephrotoxicity in about one-third of patients undergoing cisplatin treatments, and because of this characteristic, its use is limited to cancer therapy (Pabla and Dong, 2008). To create models of kidney damage in rats and mice, cisplatin is mainly used (Mishra et al., 2004; Pabla and Dong, 2008; Pinches et al., 2012).

Diclofenac reduces UA transport by interfering with *p*-aminohippuric acid (PAH) channels and causes kidney injury in chickens (Naidoo and Swan, 2009). Chicken renal tubular cells are susceptible to the toxic effects of diclofenac due to the inhibition of OAT/PAH transporters (Naidoo and Swan, 2009).

After two models of kidney damage were created in chickens, the levels of renal damage were scored by histopathological analyses and compared with gene expression levels in the kidney to identify candidate renal biomarkers.

2. Materials and Methods

2.1. Animal Experiments

All experimental protocols were approved by the Laboratory Animal Care and Use Committee of Hokkaido University (approval number: 14-0119), and the animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals of Hokkaido University, Graduate School of Veterinary Medicine, which conforms to the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The endpoints were greater than 20% weight loss compared to pre-injection weight or severe clinical symptoms, such as repeated vomiting or diarrhea. Euthanasia was carried out by isoflurane and carbon dioxide overdose (Abbott Laboratories, Chicago, IL, USA). All efforts were made to minimize animal suffering. The health of the animals wasmonitored twice daily.

2.2. Experimental Design

Male white leghorn chickens (*Gallus gallus domesticus*) ($n = 15$, 10 weeks of age, body weight: 1.2–1.4 kg) were purchased from Hokudo Co., Ltd. (Tokyo, Japan) or Sankyo Labo Service Corporation, and housed under conditions of constant temperature (20°C ± 2°C) and humidity (40% ± 10%), with a 12:12-hour light:dark cycle and given food and water *ad libitum*. The birds were acclimatized to the environment for one week before commencement of the experiments. Plasma was collected one week after arrival but prior to drug administration, which was initiated one week later. Birds were assigned randomly to one of four groups and received an injection of either 20% dimethyl sulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan) diluted in saline or diclofenac sodium (Tokyo

Chemical Industry Co., Tokyo, Japan) diluted in 20% DMSO into the pectoral muscle once daily in the morning for four consecutive days, or a single injection of cisplatin (Wako Pure Chemical Industries, Osaka, Japan) diluted in saline into the basilic vein. The following groups were considered: control group (n = 3, IDs: Cont.-1, -2, -3) injected with 20% DMSO; diclofenac sodium group A (1.5 mg/kg body weight) (n = 4, IDs: A-1, -2, -3, -4); diclofenac sodium group B (2.0 mg/kg body weight) (n = 4, IDs: B-1, -2, -3, -4); and cisplatin group (3.5 mg/kg body weight) (n = 4, IDs: C-1, -2, -3, -4). Drug doses were established to develop a specific avian model of renal damage based on the results of preliminary experiments and previous reports of diclofenac (Jain et al., 2009; Mohan et al., 2012; Naidoo et al., 2007) or cisplatin injection (Cacini and Fink, 1995; Filippich et al., 2001). Cisplatin-treated chickens were anesthetized on the third day because of severe diarrhea. Diclofenac-treated chickens were anesthetized on the fifth day, consistent with published reports.

2.3. Blood Collection

Every morning, prior to feeding, blood samples were collected from the basilic vein using a 23- or 24-gauge (G) needle and heparin-containing syringe from the second day of administration forward (24, 48, 72, and 96 hours after first exposure in diclofenac groups and control group, 24 and 48 hours after injection in the cisplatin group). After the initiation of drug administration, blood samples were collected before the injection for that day. Whole blood was stored on ice after collection, and plasma was prepared by centrifuging at 1630 × g for 20 minutes at 4°C within 2 hours of collection. Plasma specimens were immediately frozen in liquid nitrogen and stored at −80°C.

2.4. UA Analysis by HPLC

Analyses were performed by high-performance liquid chromatography separation and ultraviolet detection (HPLC-UV, 20A series; Shimadzu, Kyoto, Japan), and the improved HPLC method of the Japan Society of Clinical Chemistry (JSCC) was used for measurements of UA. Briefly, 25 μL aliquots of plasma specimens or standards were mixed with 225 μL of 0.3 mol/L perchloric acid and cooled on ice for 30 minutes. The samples were mixed again by vortexing and centrifuged at 750 × *g* for 10 minutes at 4°C. Aliquots (150 μL) of the supernatants were collected and centrifuged again at 750 × *g* for 10 minutes at 4°C. Then, 50 μL aliquots of the supernatants were collected and placed into HPLC vials, followed by the addition of 50 μL of 10 mmol/L ammonium acetate. For HPLC calibration, 100.3 mg UA (Wako Pure Chemical Industries) was dissolved in 0.01 mol/L lithium carbonate in a final volume of 100 mL (1 g/L). Standards (1.25, 2.5, 5, 10, 25, and 50 mg/L) were diluted with deionized distilled water. A UV detector set at 284 nm was used to monitor the effluent. Mobile phase A consisted of 10 mmol/L ammonium acetate (pH 4.8), and phase B consisted of 100% methanol. An Inertsil ODS-3 column (2.1 mm × 150 mm: GL Sciences, Inc., Tokyo, Japan) was used for separation at a flow rate of 0.2 mL/minute, and the injection volume was 5 μL. The R^2 value of the linear regression line was 0.998.

2.5. Tissue Sample Collection

Chickens were euthanized with an overdose of isoflurane (Abbott Laboratories) and carbon dioxide. After euthanasia, four different tissues were dissected: kidney, liver, lung, and heart. Body, liver, and kidney weights were measured. The excised tissues were cut into small pieces and placed into RNAlater® (Sigma-Aldrich) at −20°C after an

overnight incubation at 4°C for microarray analysis, flash frozen in liquid nitrogen and stored at −80°C, or stored in 10% neutral-buffered formalin (Mildform®10N; Wako Pure Chemical Industries) for histopathological analysis.

2.6. Histopathological Analysis

Paraffin-embedded kidney sections were stained with periodic acid-Schiff. For scoring of renal damage, the ratio of the outer/lumenal areas at the cross-section of the tubules was measured using Axiovision Rel 4.8 software (Zeiss, Germany). In addition, three stages of histopathological alterations of the kidney (Salamat et al., 2014) were used as a reference.

2.7. RNA Isolation and Microarray Analysis

Total RNA was extracted from the cranial division of the kidney. Chickens were selected according to the level of renal damage by histopathological analyses using NucleoSpin® RNA II (Takara Bio Inc., Tokyo, Japan).

Microarray analysis was performed by Hokkaido System Science Co., Ltd. (Hokkaido, Japan). First, RNA was quantified using the NanoDrop 1000 (Thermo Scientific, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer series II (Agilent Technologies, Palo Alto, CA, USA). In brief, total RNA was reverse-transcribed into cDNA, synthesized with cyanine 3 (Cy3)-labeled cRNA, and amplified using a Low Input Quick Amp Labeling kit (Agilent Technologies, Palo Alto, CA), RNeasy mini spin columns (Qiagen, Valencia, CA, USA), and a Gene Expression Hybridization kit (Agilent Technologies, Palo Alto, CA). The Cy3-labeled cRNA probes were hybridized onto a 4 × 44 K Agilent custom chicken oligo microarray. The arrays were scanned by an Agilent

Technologies Microarray Scanner, extracted, and analyzed by Agilent Feature Extraction 12.0.3.1 (Agilent Technologies, Palo Alto, CA).

Microarray data were normalized by GeneSpring GX (Agilent Technologies, Palo Alto, CA) with a 75 percentile shift per chip. Data filtration was performed according to AFE quantification flags (gIsSaturated: 0, gIsFeatNonUnifOL: 0, gIsBGNonUnifOL: 0, gIsFeatPopnOL: 0, gIsBGPopnOL: 0, and gIsWellAboveBG: 1), resulting in 29,826 valid probes. To analyze expression pattern changes, we considered significant difference criteria to be greater than a fivefold change in signal intensity at all levels of renal damage (K7–K10). For differentially expressed genes, Gene Ontology (GO) analysis with Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/) (Huang et al., 2009; Huang da W, Sherman BT, 2009) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (http://www.genome.jp/kegg/) (Kanehisa et al., 2016; Ogata et al., 1999) were performed. For GO and KEGG pathway analyses, probe IDs were converted into Entrez Gene IDs, according to their accession information using biomaRt, DAVID, and the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/) (Durinck et al., 2005; Smedley et al., 2015). Hierarchical clustering analyses using Spearman's correlation were performed for the differentially expressed genes with R software (ver. 3.3.2).

To identify the biomarkers, genes were selected that showed expression level increases consistent with the degree of renal injury and a greater than fivefold change at all levels of renal injury. Moreover, genes that had been reported as human biomarkers were also investigated in this study.

2.8. Quantitative Real-Time PCR

Total RNA was reverse-transcribed using Rever Tra Ace (Toyobo, Osaka, Japan), according to the manufacturer's instructions. Gene-specific quantitative real-time PCR primers (Table 2) were synthesized by Sigma-Aldrich (Tokyo, Japan). The efficiency of all primers was 97%–100%. Quantitative real-time PCR was performed with the Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, CA). The 10 μL reaction mixtures consisted of the Fast SYBR Green Master Mix (Applied Biosystems), forward and reverse primers, and cDNA derived from total RNA. Phosphoglycerate kinase 1 (PKG1) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) genes were used as reference genes (Olias et al., 2014). All samples were analyzed in duplicate using the following protocol: 95°C for 20 seconds, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. At the end of each PCR run, a melting curve analysis was performed in the range of 60°C–95°C. Measurements of genes were performed in duplicate. The expression of each gene was normalized with respect to the average expression levels of PKG1 and YWHAZ, and calculated relative to control levels using the comparative threshold cycle (Ct) method. PCR products were confirmed as single fragments by electrophoresis and direct sequencing.

2.9. Statistical Analysis

To compare the results of biochemical analyses both before and after drug administration, body and tissue weights of controls and treatment groups were analyzed by Steel's test. To compare renal damage scores and glycan levels among chickens, data were analyzed using Spearman's rank correlation coefficient. Statistical analyses were

performed using JMP Pro 13 (SAS Institute, Cary, NC). For all analyses, a *P* value less than 0.05 was deemed statistically significant.

3. Results

3.1. Clinical Signs

All cisplatin-treated chickens had diarrhea and became lethargic from the second day of administration. In diclofenac-treated chickens, one chicken (ID: A-1) had diarrhea and showed lethargic from the fourth day of administration; another chicken (B-1) had white-colored diarrhea from the second day of injection. Another chicken (B-2) had diarrhea from the second day of administration but eventually recovered. The other chickens generally appeared normal. In the diclofenac group, one chicken (B-1) died due to a severe renal lesion on the third day.

3.2. UA Levels

UA levels were totally dependent on the individual, even those in the same group (Table 1). In diclofenac groups, B-1 and B-2 chickens showed abnormal UA levels 24 hours after the first administration. In addition, UA levels in A-1 and B-4 chickens increased after 72 hours. In the case of B-2 and B-4 chickens, UA levels decreased after 96 hours compared to 72 hours. All chickens in the cisplatin group had high UA levels after 48 hours.

3.3. Gross Pathology

The kidneys of A-1, B-1, and B-2 chickens in the diclofenac-treated group and all cisplatin-treated chickens were pale with evidence of hypertrophic renal changes (Fig. 1). Chickens in the control group did not show any gross pathological changes.

3.4. Histopathological Analysis

Kidney histopathology completely differed between the diclofenac and cisplatin groups. Although renal damage levels differed in each individual, diclofenac-treated chickens showed interstitial nephritis and tubular failure, whereas cisplatin-treated chickens showed tubular necrosis with protein casts evident in the tubules. A-1 and B-1 chickens in the diclofenac-treated group, and C-1 and C-3 chickens in the cisplatin-treated group, showed severe renal lesions. There were degenerative and necrotic lesions in the proximal and distal tubules, and glomerulus, including unclear structures in the nucleus and cytoplasm, infiltration of heterophils in the tubulointerstitium and proximal tubular lumen, and dilation of the proximal and distal tubular lumens (Fig. 2). B-2, C-2, and C-4 chickens also showed severe lesions in the kidney, although the degree of injury was milder than that observed in the A-1, B-1, C-1, and C-3 chickens (Fig. 2). Chicken B-4 showed mild degenerative lesions, such as slight dilations of the proximal and distal tubular lumens, and several proximal tubular lumens showed evidence of dead cells in a very limited area. No other chickens showed any significant lesions.

From these results, renal lesions were given scores ranging from K0 (no lesions) to K10 (most severe), according to the ratio of the outer/lumen areas at the cross-section of the tubules, which was measured using Axiovision Rel 4.8 software (Zeiss, Germany) as follows: K0 (the ratio of outer/lumen area was >13); K1 (10–12); K2 (8–9); K3 (7–8); K4 (6–7); K5 (5–6); K6 (4–5); K7 (3); K8 (3); K9 (3); and K10 (<2). Levels of renal damage for each chicken were defined as follows; K0 (controls); K1 (A-2, A-3, A-4, B-3); K2 (B-4); K4 (B-2); K6 (C-2); K7 (C-4); K8 (A-1); K9 (B-1); and K10 (C-1, C-3). For microarray analysis, kidneys were pooled (five groups) according to the drugs and level of damage as follows: Controls (Cont.-1, -2, -3); Diclofenac-mild (B-2, B-4);

Diclofenac-severe (A-1, B-1); Cisplatin-mild (C-2, C-4); and Cisplatin-severe (C-1, C-3). The other tissues did not show remarkable histopathological changes.

3.5. Microarray Analysis

One hundred thirty-five genes had a greater than fivefold increase in expression at all moderate and severe levels of renal injury (K4–K10). Of all these genes, 59 were classified by DAVID. Differentially expressed genes between the control and damaged groups all responded to external stimuli ($p < 0.05$). In pathway analysis, no clustering was detected.

Hierarchical clustering analyses revealed that gene expression changes clustered into two groups: K5 and lower, and greater than K5 (Fig. 3). The more damaged cluster was also divided into two groups: diclofenac injected and cisplatin injected.

To control for chemical effects, genes were selected that showed increased expression levels that were consistent with the level of renal injury. Microarray analysis showed that four genes had increased expression levels proportional to the level of renal injury with a fivefold change in expression compared to those with controls (K0) (Table 3).

3.6. Quantitative Real-Time PCR

To identify biomarker candidates in bird species, quantitative real-time PCR was performed. Microarray analysis indicated that the vanin-1 (VNN1) gene showed promise as an avian renal biomarker. The VNN1 gene has also been reported as a marker of kidney damage in a rat model of type 1 diabetic nephropathy (Fugmann et al., 2011). Therefore, the expression levels of the VNN gene in all chickens were quantified using quantitative

real-time PCR. The results of comparative Ct showed that VNN1 levels increased proportionally to the level of renal damage in both diclofenac- and cisplatin-treated chickens. Moreover, VNN1 levels were altered even in slightly damaged kidneys, such as in the kidneys of A-2, A-3, A-4, and B-3 chickens, which each had renal damage scores of K1 (Fig. 4).

4. Discussion

From both UA levels and histopathological analyses of the kidneys, renal damage was confirmed in chickens in both treatment groups, although drug sensitivity differed in each individual. Moreover, histopathological changes in the kidney showed that characteristics of renal damage differed between the diclofenac and cisplatin groups. Therefore, two models of renal damage (ATN and AIN) were created in chickens in this study.

Microarray analysis was performed to profile the differentially expressed genes within the renal lesions. GO analysis suggested that genes related to responses to external stimuli were differentially expressed in moderately and severely damaged kidneys (K4–K10). This expression pattern is similar to that observed in humans. No clusters were found by pathway analysis. Hierarchical clustering analyses indicated that differentially expressed genes clustered by damage level: K0–K4 and K6–K10. However, in each cluster, effects of diclofenac and cisplatin differed. This finding suggests that most differentially expressed genes are affected by chemicals and a few are also dependent on the extent of renal damage.

In this study, the expression of four genes—VNN1, anillin actin-binding protein (ANLN), gastrin-releasing peptide (GRP), and mini-chromosome maintenance complex component 10 (MCM10)—are dependent on the extent of renal damage and increased more than fivefold in mildly damaged kidneys. Mutations in ANLN cause focal segmental glomerulosclerosis (FSGS) (Gbadegesin et al., 2014), and GRP is a growth factor for renal cell carcinoma (RCC) (Heuser et al., 2005). MCM genes are overexpressed in renal cancers (Ha et al., 2004; Rodins et al., 2002; Taran et al., 2011).

To identify biomarkers of kidney injury, the VNN1 gene, whose expression increased proportionally to the extent of renal damage and was induced more than 15-fold in mildly damaged kidneys, was investigated. From the results of quantitative real-time PCR, VNN1 showed promise as a marker of renal damage, including slight renal failure, in both diclofenac- and cisplatin-treated chickens. Four chickens (A-2, A-3, A-4, and B-3) had normal UA levels and few histopathological changes in their kidneys. However, their VNN levels were increased compared to controls. Therefore, VNN1 could serve as an earlier biomarker of kidney injury than UA, as it can be detected in conditions of mild renal damage. Indeed, VNN1 is a potential biomarker of nephrotoxicant-induced renal injury in both humans (Hosohata et al., 2011; Hosohata et al., 2012) and rats (Fugmann et al., 2011). The current study showed that VNN1 levels were also altered in chickens, consistent with renal damage scores. Moreover, increases in VNN1 levels correlate with levels of renal damage in humans and rats. Therefore, this candidate marker may successfully reflect kidney injury in avians.

Although further studies are needed, VNN may serve as a successful early biomarker of renal damage in avians. If kidney injury is detected at an early stage, it could be treated earlier. Moreover, many medicines are nephrotoxic and require kidney function monitoring to be used effectively. In addition, renal biomarkers could be used to monitor chemical pollution that cause kidney injury, such as exposure to metals (e.g., Pb, Cd, Hg) and medicines (e.g., diclofenac).

5. Conclusions

Transcriptome analysis suggests that AIN and ATN affect gene expression via different mechanisms. However, expression levels of the VNN1 gene are dependent on the extent of renal damage and can even be detected in cases of mild damage. It could thus serve as a useful biomarker for early stage renal lesions in birds. Early renal biomarkers in birds could contribute to conservation efforts for avian species.

Table 1. Uric acid (UA) level (mg/kg) in all chickens.

ID	Treatment	Pre-injection	24h	48h	72h	96h
Cont.-1	Control	4.6	3.3	2.5	2.5	3.4
Cont.-2	Control	2.9	2.8	3.6	3.5	3.1
Cont.-3	Control	4.7	3.4	1.1	2.2	2.1
A-1	Diclofenac 1.5 mg/kg	3.2	4.0	3.4	277.0	184.9
A-2	Diclofenac 1.5 mg/kg	5.0	3.2	5.0	5.5	4.3
A-3	Diclofenac 1.5 mg/kg	3.7	2.6	2.1	4.2	2.5
A-4	Diclofenac 1.5 mg/kg	5.8	5.1	4.7	4.9	3.6
B-1	Diclofenac 2.0 mg/kg	2.9	161.0	253.4	- *	- *
B-2	Diclofenac 2.0 mg/kg	4.5	41.9	32.8	20.4	11.1
B-3	Diclofenac 2.0 mg/kg	3.8	4.5	5.6	4.3	3.1
B-4	Diclofenac 2.0 mg/kg	3.8	3.0	6.3	11.3	5.9
C-1	Cisplatin 3.5 mg/kg	3.7	7.1	70.6	-	-
C-2	Cisplatin 3.5 mg/kg	3.0	9.5	90.2	-	-
C-3	Cisplatin 3.5 mg/kg	3.6	7.2	53.5	-	-
C-4	Cisplatin 3.5 mg/kg	3.7	8.1	93.7	-	-

* B1 chicken was dead on the 3rd day of administration.

Table 2. Primers used in this study.

Target gene		Nucleotide sequence 5' to 3'	Fragment length (bp)	Reference
VNN1	Forward	TTACGTACTGGGCGCATTTG	140	designed in this study
	Reverse	CATGGCAAACTTGGTCTGTG		
PGK1	Forward	AAAGTTCAGGATAAGATCCAGCTG	167	Philipp et al. 2014
	Reverse	GCCATCAGGTCCTTGACAAT		
YWHAZ	Forward	GTGGAGCAATCACAACAGGC	222-224	Philipp et al. 2014
	Reverse	GCGTGCGTCTTTGTATGACTC		

Table 3. Gene induction shown by microarray analysis. VNN1: vanin-1; ANLN: anillin actin-binding protein; GRP: gastrin-releasing peptide; MCM10: mini-chromosome maintenance complex component 10.

	Induction ratio compared to K0					
Gene name	K0	K2	K4	K6-7	K7-9	K9-10
VNN1	1	15.74424	27.57141	51.04239	111.5134	119.5326
ANLN	1	7.449375	13.32174	16.19249	26.72836	26.8043
GRP	1	6.583954	10.6525	12.98904	17.54708	24.90333
MCM10	1	5.340534	5.514153	5.77615	7.039732	7.132706

Fig 1. Gross pathological features of kidneys in diclofenac- or cisplatin-treated, or control chickens. The chickens treated with diclofenac (A-1, B-1, 2) or cisplatin (C-1, 2, 3, 4) showed pale and hypertrophic kidneys compared with the controls.

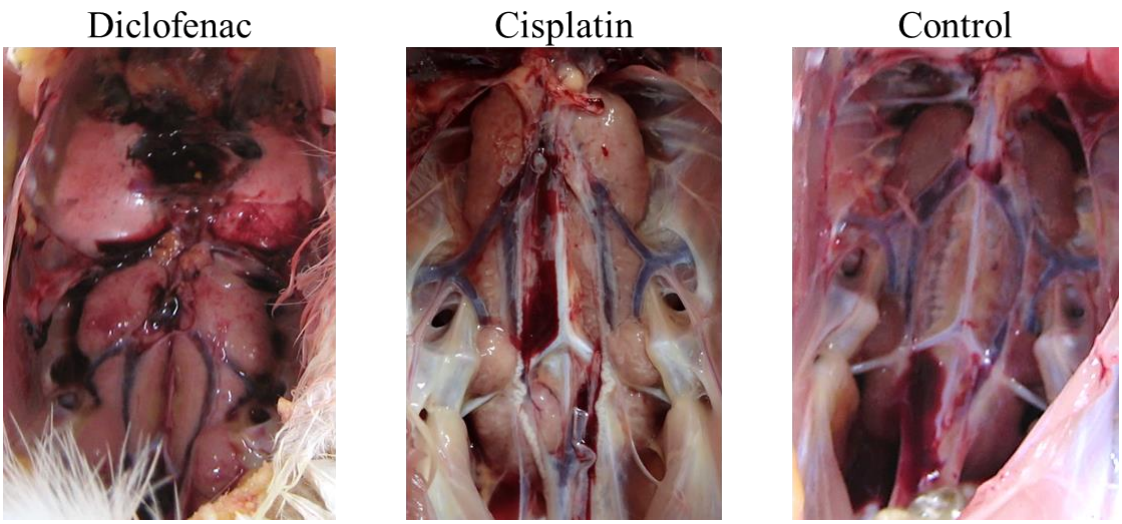


Fig 2. Histopathological features of the kidneys of diclofenac-treated, cisplatin-treated, and control chickens. The chickens treated with diclofenac showed AIN and with cisplatin had ATN. Control chickens did not show the histopathological changes.

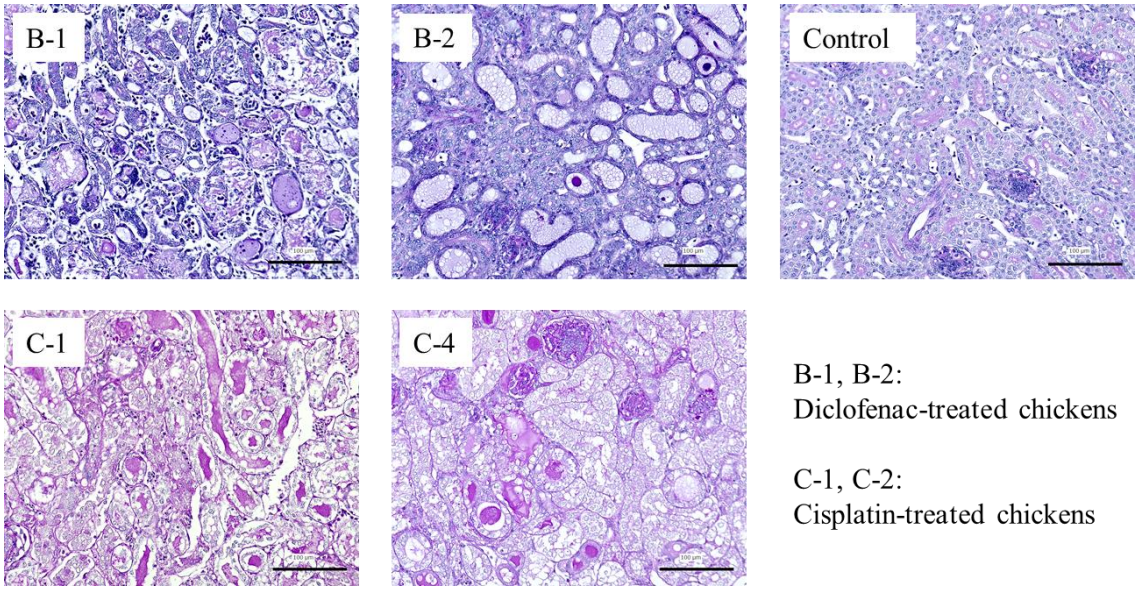


Fig. 3. Heatmap of differentially expressed genes in chickens with renal damage scores of K0 and K4–K10.

The genes were clustered into two groups; one is severe damage and the other is none to moderate damage.

C: cisplatin group, D: diclofenac group

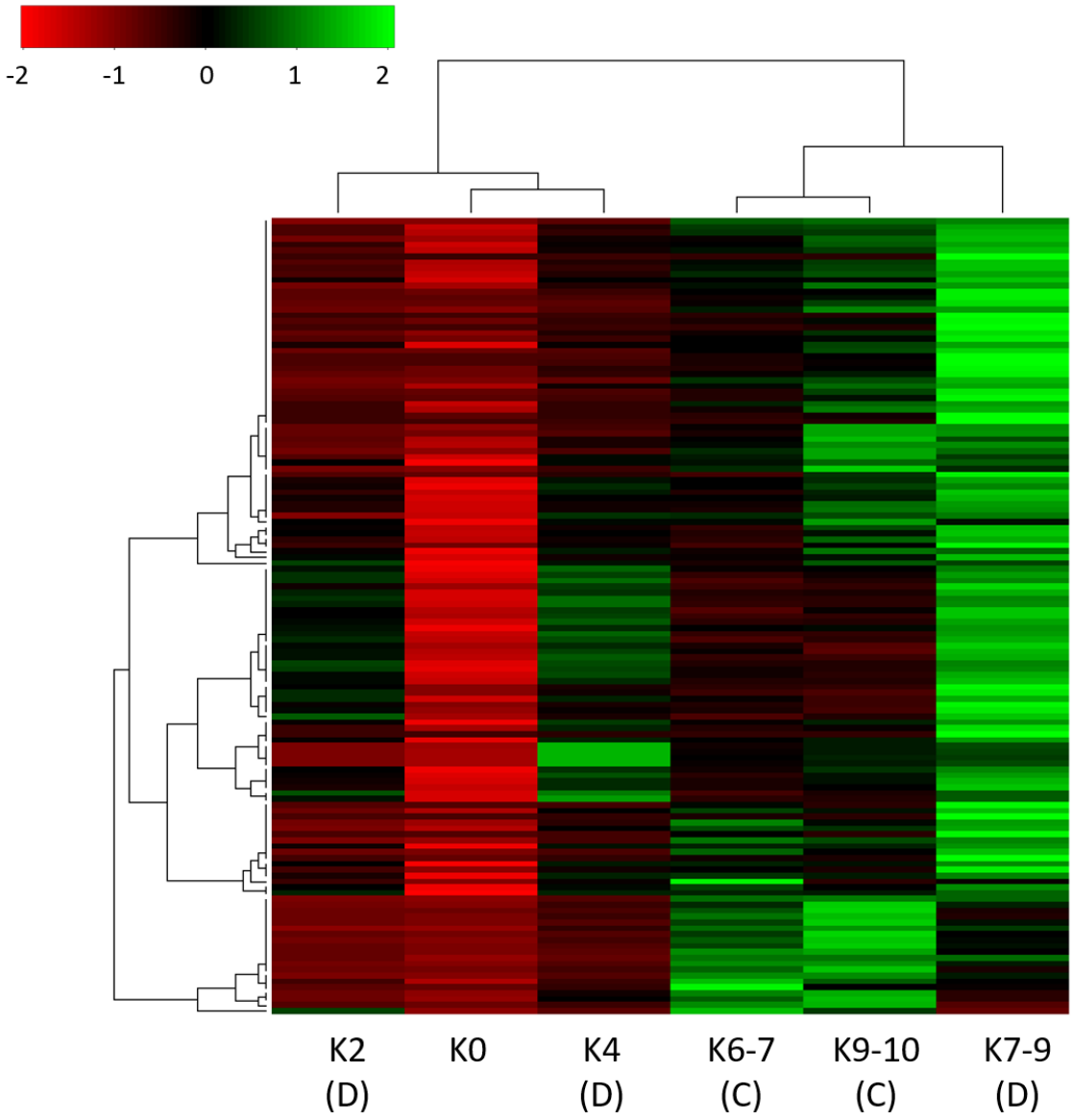
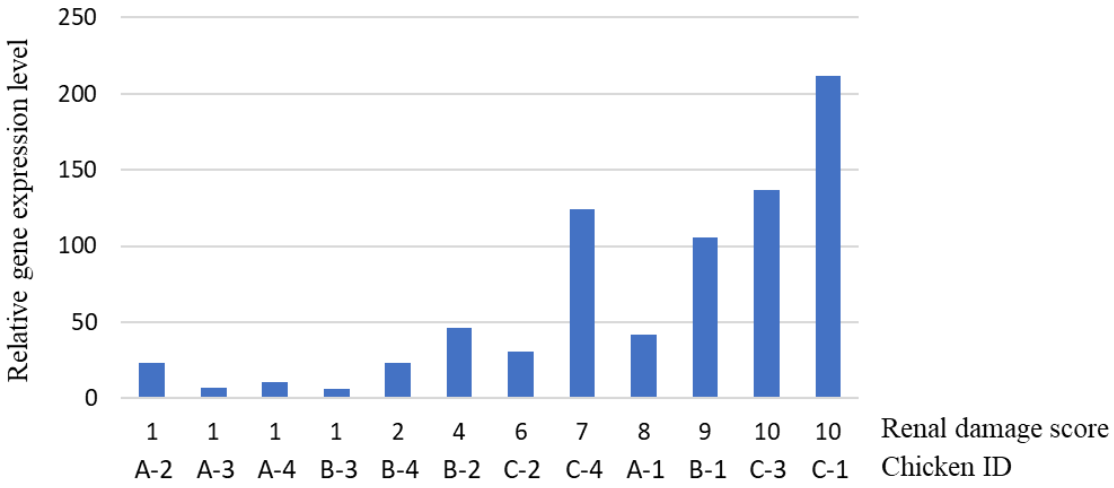


Fig. 4. Gene expression levels of VNN1 by quantitative real-time PCR (Comparative Ct).



References

Arany, I., Safirstein, R.L., 2003. Cisplatin nephrotoxicity, in: Seminars in Nephrology. Elsevier, pp. 460–464.

Cacini, W., Fink, I.M., 1995. Toxicity and excretion of cisplatin in the avian kidney. Comp. Biochem. Physiol. Part C Pharmacol. Toxicol. Endocrinol. 111, 343–350.

Cruz, D.N., de Cal, M., Garzotto, F., Perazella, M.A., Lentini, P., Corradi, V., Piccinni, P., Ronco, C., 2010. Plasma neutrophil gelatinase-associated lipocalin is an early biomarker for acute kidney injury in an adult ICU population. Intensive Care Med. 36, 444–451.

Dharnidharka, V.R., Kwon, C., Stevens, G., 2002. Serum cystatin C is superior to serum creatinine as a marker of kidney function: a meta-analysis. Am. J. Kidney Dis. 40, 221–226.

Durinck, S., Moreau, Y., Kasprzyk, A., Davis, S., De Moor, B., Brazma, A., Huber, W., 2005. BioMart and Bioconductor: A powerful link between biological databases and microarray data analysis. Bioinformatics 21, 3439–3440. https://doi.org/10.1093/bioinformatics/bti525

Filippich, L.J., Bucher, A.M., Charles, B.G., Sutton, R.H., 2001. Intravenous cisplatin administration in sulphur-crested cockatoos (*Cacatua galerita*): Clinical and pathologic observations. J. Avian Med. Surg. 15, 23–30.

Fugmann, T., Borgia, B., Révész, C., Godó, M., Forsblom, C., Hamar, P., Holthöfer, H., Neri, D., Roesli, C., 2011. Proteomic identification of vanin-1 as a marker of kidney damage in a rat model of type 1 diabetic nephropathy. Kidney Int. 80, 272–281.

Gbadegesin, R.A., Hall, G., Adeyemo, A., Hanke, N., Tossidou, I., Burchette, J., Wu, G., Homstad, A., Sparks, M.A., Gomez, J., Jiang, R., Alonso, A., Lavin, P., Conlon, P., Korstanje, R., Stander, M.C., Shamsan, G., Barua, M., Spurney, R., Singhal, P.C., Kopp, J.B., Haller, H., Howell, D., Pollak, M.R., Shaw, A.S., Schiffer, M., Winn, M.P., 2014. Mutations in the Gene That Encodes the F-Actin Binding Protein Anillin Cause FSGS. J. Am. Soc. Nephrol. 25, 1991–2002. https://doi.org/10.1681/ASN.2013090976

Green, R.E., Newton, I.A.N., Shultz, S., Cunningham, A.A., Gilbert, M., Pain, D.J., Prakash, V., 2004. Diclofenac poisoning as a cause of vulture population declines across the Indian subcontinent. J. Appl. Ecol. 41, 793–800.

Ha, S.-A., Shin, S.M., Namkoong, H., Lee, H., Cho, G.W., Hur, S.Y., Kim, T.E., Kim, J.W., 2004. Cancer-associated expression of minichromosome maintenance 3 gene in several human cancers and its involvement in tumorigenesis. Clin. Cancer Res. 10, 8386–95. https://doi.org/10.1158/1078-0432.CCR-04-1029

Han, W.K., Bailly, V., Abichandani, R., Thadhani, R., Bonventre, J. V, 2002. Kidney Injury Molecule-1 (KIM-1): a novel biomarker for human renal proximal tubule injury. Kidney Int. 62, 237–244.

Harr, K.E., 2002. Clinical chemistry of companion avian species: a review. Vet. Clin. Pathol. 31, 140–151.

Heuser, M., Schlott, T., Schally, A. V, Kahler, E., Schliephake, R., Laabs, S.O., Hemmerlein, B., 2005. Expression of gastrin releasing Peptide receptor in renal cell carcinomas: a potential function for the regulation of neoangiogenesis and microvascular perfusion. J. Urol. 173, 2154–2159. https://doi.org/10.1097/01.ju.0000158135.26893.bc

Hosohata, K., 2016. Role of Oxidative Stress in Drug-Induced Kidney Injury. Int. J. Mol. Sci. 17, 1826.

Hosohata, K., Ando, H., Fujimura, A., 2012. Urinary vanin-1 as a novel biomarker for early detection of drug-induced acute kidney injury. J. Pharmacol. Exp. Ther. 341, 656–662.

Hosohata, K., Ando, H., Fujiwara, Y., Fujimura, A., 2011. Vanin-1; a potential biomarker for nephrotoxicant-induced renal injury. Toxicology 290, 82–88.

Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009. Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. 37, 1–13. https://doi.org/10.1093/nar/gkn923

Huang da W, Sherman BT, L.R., 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57.

Ichii, O., Otsuka, S., Sasaki, N., Namiki, Y., Hashimoto, Y., Kon, Y., 2012. Altered expression of microRNA miR-146a correlates with the development of chronic renal inflammation. Kidney Int. 81, 280–292.

Jain, T., Koley, K.M., Vadlamudi, V.P., Ghosh, R.C., Roy, S., Tiwari, S., Sahu, U., 2009. Diclofenac-induced biochemical and histopathological changes in white leghorn birds (*Gallus domesticus*). Indian J. Pharmacol. 41, 237–241. https://doi.org/10.4103/0253-7613.58515

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., Tanabe, M., 2016. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res. 44, D457–D462. https://doi.org/10.1093/nar/gkv1070

Lierz, M., 2003. Avian renal disease: pathogenesis, diagnosis, and therapy. Vet. Clin. North Am. Exot. Anim. Pract. 6, 29–55.

Mishra, J., Mori, K., Ma, Q., Kelly, C., Barasch, J., Devarajan, P., 2004. Neutrophil gelatinase-associated lipocalin: a novel early urinary biomarker for cisplatin nephrotoxicity. Am. J. Nephrol. 24, 307–315.

Mohan, K., Jayakumar, K., Narayanaswamy, H.D., Manafi, M., Pavithra, B.H., 2012. An initial safety assessment of hepatotoxic and nephrotoxic potential of intramuscular ketoprofen at single repetitive dose level in broiler chickens. Poult. Sci. 91, 1308–1314.

Naidoo, V., Duncan, N., Bekker, L., Swan, G., 2007. Validating the domestic fowl as a model to investigate the pathophysiology of diclofenac in *Gyps* vultures. Environ. Toxicol. Pharmacol. 24, 260–266.

Naidoo, V., Swan, G.E., 2009. Diclofenac toxicity in Gyps vulture is associated with decreased uric acid excretion and not renal portal vasoconstriction. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 149, 269–274.

Ogata, H., Goto, S., Sato, K., Fujibuchi, W., Bono, H., Kanehisa, M., 1999. KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 27, 29–34. https://doi.org/10.1093/nar/27.1.29

Olias, P., Adam, I., Meyer, A., Scharff, C., Gruber, A.D., 2014. Reference genes for quantitative gene expression studies in multiple avian species. PLoS One 9, 26–28. https://doi.org/10.1371/journal.pone.0099678

Pabla, N., Dong, Z., 2008. Cisplatin nephrotoxicity: mechanisms and renoprotective strategies. Kidney Int. 73, 994–1007.

Pinches, M., Betts, C., Bickerton, S., Burdett, L., Thomas, H., Derbyshire, N., Jones, H.B., Moores, M., 2012. Evaluation of novel renal biomarkers with a cisplatin

model of kidney injury: gender and dosage differences. Toxicol. Pathol. 40, 522–533.

Rodins, K., Cheale, M., Coleman, N., Fox, S.B., 2002. Minichromosome maintenance protein 2 expression in normal kidney and renal cell carcinomas: relationship to tumor dormancy and potential clinical utility. Clin. Cancer Res. 8, 1075–81.

Salamat, N., Etemadi-Deylami, E., Movahedinia, A., Mohammadi, Y., 2014. Heavy metals in selected tissues and histopathological changes in liver and kidney of common moorhen (*Gallinula chloropus*) from Anzali Wetland, the south Caspian Sea, Iran. Ecotoxicol. Environ. Saf. 110, 298–307.

Shideman, J.R., Evans, R.L., Bierer, D.W., Quebbemann, A.J., 1981. Renal venous portal contribution to PAH and uric acid clearance in the chicken. Am. J. Physiol. Physiol. 240, F46–F53.

Smedley, D., Haider, S., Durinck, S., Pandini, L., Provero, P., Allen, J., Arnaiz, O., Awedh, M.H., Baldock, R., Barbiera, G., Bardou, P., Beck, T., Blake, A., Bonierbale, M., Brookes, A.J., Bucci, G., Buetti, I., Burge, S., Cabau, C., Carlson, J.W., Chelala, C., Chrysostomou, C., Cittaro, D., Collin, O., Cordova, R., Cutts, R.J., Dassi, E., Di Genova, A., Djari, A., Esposito, A., Estrella, H., Eyras, E., Fernandez-Banet, J., Forbes, S., Free, R.C., Fujisawa, T., Gadaleta, E., Garcia-Manteiga, J.M., Goodstein, D., Gray, K., Guerra-Assunção, J.A., Haggarty, B., Han, D.J., Han, B.W., Harris, T., Harshbarger, J., Hastings, R.K., Hayes, R.D., Hoede, C., Hu, S., Hu, Z.L., Hutchins, L., Kan, Z., Kawaji, H., Keliet, A., Kerhornou, A., Kim, S., Kinsella, R., Klopp, C., Kong, L., Lawson, D., Lazarevic, D., Lee, J.H., Letellier, T., Li, C.Y., Lio, P., Liu, C.J., Luo, J., Maass, A., Mariette, J., Maurel, T., Merella, S., Mohamed, A.M., Moreews, F., Nabihoudine, I.,

Ndegwa, N., Noirot, C., Perez-Llamas, C., Primig, M., Quattrone, A., Quesneville, H., Rambaldi, D., Reecy, J., Riba, M., Rosanoff, S., Saddiq, A.A., Salas, E., Sallou, O., Shepherd, R., Simon, R., Sperling, L., Spooner, W., Staines, D.M., Steinbach, D., Stone, K., Stupka, E., Teague, J.W., Dayem Ullah, A.Z., Wang, J., Ware, D., Wong-Erasmus, M., Youens-Clark, K., Zadissa, A., Zhang, S.J., Kasprzyk, A., 2015. The BioMart community portal: An innovative alternative to large, centralized data repositories. Nucleic Acids Res. 43, W589–W598. https://doi.org/10.1093/nar/gkv350

Swan, G.E., Cuthbert, R., Quevedo, M., Green, R.E., Pain, D.J., Bartels, P., Cunningham, A.A., Duncan, N., Meharg, A.A., Oaks, J.L., 2006. Toxicity of diclofenac to Gyps vultures. Biol. Lett. 2, 279–282.

Taran, K., Sitkiewicz, A., Andrzejewska, E., Kobos, J., 2011. Minichromosome maintenance 2 (MCM2) is a new prognostic proliferative marker in Wilms tumour. Pol. J. Pathol. 62, 84–88.

Tully, T.N., Dorrestein, G.M., Jones, A.K., 2009. Handbook of avian medicine. Elsevier/Saunders.

Yasuda, Y., Cohen, C.D., Henger, A., Kretzler, M., 2006. Gene expression profiling analysis in nephrology: Towards molecular definition of renal disease. Clin. Exp. Nephrol. 10, 91–98. https://doi.org/10.1007/s10157-006-0421-z

SUMMARY

The ultimate goal of this research is a contribution to the conservation of birds. The studies presented here focused on chemical pollution and renal damage to birds because many birds have died from these causes. I elucidated the Pb contamination situation and the causes of Pb exposure in birds of prey and waterbirds throughout Japan, elucidated the metal accumulation characteristics in seabirds, and identified a candidate novel kidney injury marker that might be useful for clinical applications and assessment of chemical exposures that cause kidney injury in birds.

In chapter 1, I report that seabirds from the Bering Sea accumulated higher Hg concentration than previously reported, suggesting the possibility of progression of Hg pollution. In addition, a high correlation was found between the nitrogen stable isotope ratio, the carbon stable isotope ratio, and the accumulated Hg in birds. In the future, as Hg pollution increases, the earliest damage might occur in seabirds at high trophic levels. Concern about the expansion of Hg pollution in the biota of the Bering Sea is focused on the contamination of not only seabirds but also other species such as whales. In addition, accumulation of Cd, As, and other metals is thought to be strongly related to food quality.

According to an initial survey of metal contamination in the birds of Teuri Island, the accumulated concentrations of metals in these birds is lower than those in the same species in other areas. This research provided basic data on seabird metal accumulation on Teuri Island, an important breeding site for migratory birds.

In chapters 2 and 3, it becomes clear that Pb contamination damage in birds

occurs throughout Japan. In Hokkaido, despite regulation of the use of Pb ammunition, there has still been damage to endangered eagles. Pb stable isotope ratios showed that illegal use of Pb ammunition is still occurring in Hokkaido. Damage to the eagles was caused by both Pb rifle bullets and Pb shot pellets. These cases are just a part of the total number, and other individuals, not discovered, have also been damaged.

Furthermore, in the area of Honshu, regulations for the use of Pb are restricted to only a limited number of areas. Pb poisoning was not regarded as much of a problem, but birds of prey such as the endangered golden eagle have been exposed to Pb, and some individuals with high concentrations of Pb, diagnosed as having Pb poisoning, were also found. In endangered species, human-induced damage is a big problem. A reduction in the number of birds of prey species, located at the top of the food chain, has an effect on the entire ecosystem.

In waterbirds, Pb shot in inside the stomach and high concentrations of Pb accumulation in the liver were confirmed. Like birds of prey, waterbirds are damaged by Pb exposure nationwide.

Therefore, more detailed investigation of Pb contamination on a national scale is required for both birds of prey and waterbirds.

In Hokkaido, damaged individual birds have appeared even after regulation of the use of Pb ammunition, and since damage has also occurred in other regions in Honshu and Shikoku, where surveys are extremely limited, Japan-wide investigation into birds' exposure to Pb and consideration of strengthening regulations on the use of Pb ammunition are required. At the same time, it is important to convey information on Pb contamination in birds and on the effectiveness of non-Pb bullets to many citizens to reduce Pb exposure in avian species.

In chapter 4, it is shown that the Pb accumulation process differs depending on bone structure and presence or absence of bone marrow. In cases of acute exposure, there is a high probability that Pb accumulation will be particularly large in trabecular bones and bones that contain bone marrow. In cases of chronic exposure, it may be that Pb concentration also increases in bones mainly composed of cortical bone.

Analysis and comparison of Pb accumulation in various bones seem to be useful for estimating whether exposure is acute or chronic, as well as the time of exposure. The elucidation of the accumulation process in bone is expected to be applied to detailed Pb contamination investigations for the future regulation of the use of Pb ammunition.

In chapters 5 and 6, two different types of kidney injury chicken models were created using two nephrotoxic drugs (diclofenac and cisplatin) to identify novel renal biomarkers. AKI (acute kidney injury) can be classified as AIN (acute interstitial nephritis) or ATN (acute tubular necrosis); diclofenac causes AIN whereas cisplatin causes ATN.

The result of comprehensive glycan analysis in the search for biomarkers showed that there was an increase in glycan expressions after administration of 14 kinds of *N*-glycans in the diclofenac group. Many of these glycans are sialylated and have a non-fucosylated structure, and five of them are confirmed to have increased expression in human renal carcinoma. However, in the cisplatin-administered group, no changes in glycans were observed, and it is thought that differences in glycan expression appeared due to differences in diseased sites in the kidney. These results suggest that glycans may be useful as markers for AIN.

In gene expression analysis, several types of genes whose expression increased in a disability-dependent manner in the diclofenac-administered group and the cisplatin-administered group were observed in the microarray analysis. Among them, the expression of VNN1 (vanin-1) increased in a nearly disability-dependent manner in both groups. In addition, VNN1 was also elevated in individuals who were diagnosed by pathological examination as having a slight degree of renal disability, and there was no change in uric acid value in these individuals. Therefore, VNN1 can rise earlier in the renal disease process than the uric acid value can.

Based on these results, VNN1 is considered an early renal injury marker candidate, reflecting both ATN and AIN. In recent years, VNN1 has also attracted attention as an early kidney injury marker in human medicine, and because of the increase in its expression in chickens as well, it is thought to be highly likely that VNN1 also reflects kidney damage in other avian species.

There are many threatened species of birds, and many of them are dying due to the influences of human activity. Pb exposure in birds is one of those problems still occurring in the world, including Japan. In California (USA), the government decided to strictly prohibit the use of Pb ammunition, and the number of California condors is incrasing gradually. The regulation of Pb ammunition in Japan is not strong enough, even in Hokkaido. We need to improve this regulation and expand it to cover all parts of Japan. To solve this problem, not only veterinarians and researchers but also governments and local residents should cooperate to share accurate information about what is happening to

wildlife, and try to understand each situation from both wildlife and human perspectives.

In addition, we have to search for a good alternative to Pb ammunition for hunting. As a substitute for Pb ammunition, Cu bullets and steel shot pellets that can be just as effective have been developed, but Pb bullets are still used. Further research about the toxicity of alternative ammunition is required. In some species, it has been found that alternative ammunition has much lower toxicity than Pb ammunition does. Although this information is very important as scientific data, sensitivity to metal exposure can be different depending on the avian species. Therefore, we have to look into species differences. Another problem is the cost of the ammunition. Although Cu rifle bullets have the same power as Pb rifle bullets, the cost of Cu bullets is more than that of Pb bullets. This is one of the reasons that some hunters are still using Pb ammunition. Furthermore, despite the scientifically demonstrated effectiveness of non-Pb bullets, it is widely believed that Pb bullets have the highest success rate in hunting. Therefore, we have to inform hunters about the effectiveness of alternative ammunition and the serious problems in birds that are caused by Pb ammunition.

Regarding the identification of novel biomarkers, especially in avian species, translational research, which delivers original investigations from laboratories to clinical practices, is extremely limited worldwide. Birds are high-risk species that are prone to kidney damage due to their unique kidney structure, and marker substances that reflect early stage kidney disease have not been found yet, despite the high utility of renal impairment markers. Therefore, establishment and validation of novel kidney injury markers for evaluation and treatment of birds' exposure to chemical substances, are required, not only in Japan but all over the world.

This study showed that VNN1 has potential to become a useful biomarker for kidney injury in birds. However, the expression level of VNN1 could have species differences even in healthy birds. Furthermore, there may be age or sex differences in VNN1 levels. Therefore, reference levels of VNN1 should be established in males, females, chicks, juveniles, and adults of various avian species.

If a novel kidney injury marker is identified in birds and a simple kidney injury diagnosis kit is developed, it will be useful in treating early-stage renal disorders, monitoring treatment with medicines (such as therapeutic drugs for Pb poisoning) that cause nephrotoxicity as a side effect, and the early detection of chemical contamination that causes renal disorders. Thus, identification of an early biomarker for kidney injury can contribute to avian medicine and conservation of wild birds.

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