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Lead exposure in raptors from Japan and source identification using Pb stable isotope ratios

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Abstract

Lead (Pb) poisoning is widespread among raptors and water birds. 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 poisoning, Raptor, Japan, Pb isotope ratios

1. Introduction

Lead (Pb) poisoning has been widespread among raptors and water birds (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. Water birds 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), 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 in Japan and to identify the sources of Pb by using stable isotope ratios. We measured hepatic and renal Pb concentrations and hepatic Pb isotope ratios of Steller's sea eagles, white-tailed sea eagles, golden eagles, 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 in Hokkaido, Honshu, and Shikoku in Japan.

2. Materials and Methods

128 *2.1. Sampling*

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

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