Behavior and Toxic Effects of Pb in a Waterfowl Model with Oral Exposure to Pb Shots: Investigating Pb Exposure in Wild Birds

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Abstract

Among wild birds, lead (Pb) exposure caused by ingestion of ammunition is a worldwide problem. We aimed to reveal the behavior and toxic effect of Pb caused by ingesting Pb shots in waterfowl. Four male, eight-week old Muscovy ducks (*Cairina moschata*) were given three Pb shots (approximately 240 mg in total) orally and then fed for 29 days after exposure, simulating a low-dose Pb exposure in wild waterfowl. During the breeding period, blood samples were collected 10 times, and fecal samples every day. Additionally, 22 fresh tissue and 6 bone samples were obtained from each duck through the dissection. Although there were no gross abnormalities, the maximum blood Pb concentration of each duck ranged from 0.6 to 3.7 mg/L, reaching a threshold concentration indicative of clinical symptoms (> 0.5 mg/L). δ-aminolevulinic acid dehydratase declined one day after exposure and remained low throughout the feeding period. Hematocrit also tended to decrease, indicating signs of anemia. The highest Pb accumulation was observed in the bones, followed by the kidneys, intestinal tracts, and liver. High Pb accumulation in the bones, which are known to have a long Pb half-life, suggested that Pb would remain in the body and possibly affect bird health beyond 28 days after exposure. Gene expression analysis showed a significant increase in the expression of the toll-like receptor-3 gene, which is involved in virus discrimination in the liver, suggesting a disruption of the immune system. Microbiota analyses showed a correlation between the blood Pb concentration and the abundances of *Lachnospiraceae* and *Ruminococcaceae*, suggesting that Pb affects lipid metabolism. These results provide fundamental data on Pb exposure in wild birds and a new perspective on the damage such exposure causes.

Keywords:

duck; lead (Pb) exposure; toxicological effects; waterfowl

1. Introduction

The survival of wild birds is threatened by environmental contaminants such as pesticides, rodenticides, and plastics (Arya et al., 2019; Rattner and Harvey, 2020; Roman et al., 2019). Lead (Pb) pollution is one of the most studied and harmful pollutions to wild birds. Various wild birds, including waterfowl and raptors, are known to suffer from the effects of Pb pollution (Plaza and Lambertucci, 2019).

Pb is a toxic non-essential metal that has no compensatory beneficial effects in living organisms. It affects a wide range of physiological and biochemical systems, including the hematopoietic, vascular, nervous, renal, immune, and reproductive systems. Pb exposure can adversely affect behavior and survival (Abadin et al., 2007; EFSA Panel on Contaminants in the Food Chain, 2010). For avian species, even a low-dose exposure to Pb is a threat that should not be disregarded. While it does not cause an immediate failure of biological functions or directly impact welfare and survival, Pb does increase mortality rate through anemia, lethargy, muscle wastage, and the loss of fat reserves, balance, and coordination. Compared to these deleterious effects, the immunosuppressive effects and disturbances to microbiota caused by Pb exposure have been given more attention. It is well known that Pb causes immunotoxicity, which increases susceptibility to disease (Hemphill et al., 1971; Youssef et al., 1996). In birds, Pb exposure can affect most components of the immune system (Vallverdú-Coll et al., 2019). Metals are thought to have deleterious effects on the microbiota composition because they are considered the primary contact for ingested metals (Duan et al., 2020). Although the effect of Pb on microbiota in avian species has been investigated (Kou et al., 2019), the effect of Pb exposure remains unclear.

It is well known that in avian species the main sources of Pb exposure are solid products, for example, Pb ammunition and fishing weights (Sarasola et al., 2018). Among avian species, and in particular waterfowl, the behavior of swallowing small stones increases their likelihood of accidental Pb ingestion. Pb shot has been recorded in the alimentary tracts of individuals in the Anatidae family in the wild (Pain et al., 2019). Amongst raptors, there is an additional important means of Pb contamination: eating Pb-contaminated animals. Further, Pb products may be retained in the ventriculus: it takes 30–35 days and 23–28 days to completely break down Pb shot (2.7 mm diameter) in the domestic duck and mallard duck, respectively (Bono and

Braca, 1973). The long-term retention of Pb products in the ventriculus suggests that Pb is released into the body over an extended period. For these reasons, birds are at high risk from exposure to solid Pb products. There has been considerable damage to avian species due to Pb shot. In the European Union, approximately 700,000 individual waterfowl die annually and an additional 2.2 million suffer sub-lethal damage due to low-dose Pb-exposure (Andreotti et al., 2018), possibly resulting in increased mortality due to anemia and impaired immune function. In Argentina, 12.8% of free-ranging ducks were found to have Pb pellets in their ventriculuses (Ferreyra et al., 2014). It is also reported that adult wild stiff-tailed ducks had a lower prevalence of ingested Pb shot than juveniles in Valencia, which is considered to be due to the foraging inexperience of juvenile birds that increases their likelihood of ingesting shot (Mateo et al., 2001). Pb exposure in wild birds is a serious and worldwide problem.

Ingested solid Pb is worn in the ventriculus, absorbed by the digestive system further downstream transported via the bloodstream, and deposited in tissues (De Francisco et al., 2003; Pain et al., 2019). This process is specific to birds since they have ventriculuses. There are limited data regarding the toxic effect of low-dose exposure in relation to avian-specific Pb ingestion and absorption. Although field studies suggest the damage to wild birds is considerable, the toxic mechanisms remain unclear. Therefore, *in vivo* experiments focused on the oral exposure of low-dose solid Pb products to demonstrate Pb erosion and absorption behavior and toxic effects are needed.

This study aims to investigate the behavior of Pb and the toxic effects caused by orally ingested low-dose Pb in waterfowl. We conducted an *in vivo* experiment simulating Pb exposure in the wild to fully identify the effects of low-dose Pb exposure in wild birds. To simulate the Pb exposure in the wild more precisely, the Muscovy duck (*Cairina Moschata*), which belongs to the Anatidae family known to suffer enormous damage in the wild, was used as a waterfowl model. In order to analyze Pb behavior, including kinetics such as Pb distribution in the intestinal tract, blood, feces and other tissues, the amount of shots retained in the ventriculuses and Pb concentrations were measured. Additionally, various analyses described in Materials and Methods were carried out to investigate toxic effects. This study provides fundamental data to help identify potential hazards of low-dose Pb-exposure in wild

birds.

2. Materials and Methods

2.1 Animals and experimental design

Animal experiments were performed in accordance with the Regulations for Animal Experiments and Related Activities at Hokkaido University. The animal experiments were approved by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) and the Institutional Animal Care and Use Committee of Hokkaido University (approval No. 18-0092).

Seven Muscovy ducks (male, eight-week-old, 3.18–3.75 kg in body weight) were divided into two groups: a control group (N = 3) and a Pb-exposed group (N = 4). Ducks were housed individually in cages with free access to water and food.

Three Pb shots (240 ± 1.7 mg in total) were orally administered to the Pb-exposure group; In a preliminary experiment, a duck exposed to one Pb shot had not shown significant change (data not shown), therefore three Pb shots, which is the lowest number of Pb shots with the range that occurred in the wild, were used in this study. Pb shot of 80 mg is used to hunt small birds and 300–500 Pb shots are released per cartridge so it is thought that wild waterfowl in hunting areas are exposed to some tablets. In many cases, actually, Pb poisoned waterfowls ingested several Pb shots in their ventriculuses in Japan. The ducks were then housed for 29 days. Blood samples were collected 10 times, and fecal samples every day. Then 23 tissue and 6 bone samples per individual were collected.

In order to increase samples from the control group, three Muscovy ducks (male, eight-week-old, 2.9–3.05 kg) were housed for four days under the same conditions, and the tissue and bone samples were collected as described above.

2.2 Analysis of Pb behavior

For measuring Pb concentrations, 60-300 mg of tissues or 18-22 mg of bones or 80-300 μL of blood or 80-150 mg of feces were used. Tissue and fecal samples were dried at 50 ℃ for 24 hours. Subsequently, samples were digested with 5 mL of 30% nitric acid and 1 mL of 30% hydrogen peroxide in a microwave digestion system (Speedwave Two; Berghof, Germany): 160 ℃ for 5 minutes, 190 ℃ for 20 minutes,

200 ℃ for 20 minutes, and 100 ℃ for 5 minutes. Then, the sample volume was made up to 10 mL. Washing protocol was carried out every after the digestion in a microwave to prevent from contamination. All chemicals and standard solutions were of analytical reagent grade (Wako Pure Chemicals Industries, Osaka, Japan). The water used in the measurement was Milli-Q. Pb concentrations were measured using an inductively coupled plasma-mass spectrometer (ICP-MS; 7700 series; Agilent Technology, Tokyo, Japan) according to the method of Yabe with some modifications (Yabe et al., 2015). The certified reference materials were shown to have 95-105% recoveries through replicate analysis. The instrument detection limit was 0.001 μg/kg.

Additionally, the weight of the shots found in the ventriculuses during the dissection was measured. Details of the methods are described in the supplementary materials.

2.3 Analysis of toxic effects

In addition to checking ducks' status and blood biochemical tests, we measured content of metallothionein in the livers and kidneys, which is known to detoxify metals (Thirumoorthy et al., 2011). The effect on the hematopoietic system was analyzed by measuring hematocrit (Ht) and the activity of δ-aminolevulinic acid dehydratase (δ-ALAD) in the bloods, which is involved in heme biosynthesis but is inhibited by Pb, possibly resulting in anemia (Rogan et al., 1986). We conducted gene expression analysis of six tissues and next-generation sequencing of fecal DNA to screen for changes in the immune system and microbiota, respectively. The expression of immune and oxidative stress genes, including interferon-gamma, (IFN-gamma), interleukin-6 (IL6), interleukin-8 (IL8), inducible nitric oxide synthase (INOS), nuclear factor-kappa B subunit 1 (NF-KB1), prostaglandin-endoperoxide synthase 2 (PTGS2), toll-like receptor 3 (TLR3), and tumor necrosis factor-alpha (TNFA), were quantified using real time PCR. Microbiota were analyzed by 16S rDNA (V4) amplicon sequencing. Detailed methods for each analysis are provided in the supplementary materials.

2.4 Data analysis

All statistical analyses were carried out using JMP Pro version 14 (SAS

Institute, Cary, NC, USA). A Wilcoxon test was performed between the control and exposed groups for each gene and tissue.

A principal components analysis (PCA) was carried out to investigate the expression of each gene and the Pb concentrations in all tissues. A Spearman's rank-order correlation coefficient was calculated between the gene expression and Pb concentration for each tissue.

To analyze microbiota, an α-rarefaction, reflected by the Shannon index, and the Chao-1 index were calculated based on the read numbers of operational taxonomic units (OTUs). A Spearman's rank-order correlation coefficient was calculated between each OUT abundance and blood Pb concentration using JMP 14.

3. Results and Discussion

3.1. The behavior of Pb from shots retained in the ventriculus

After ingestion, Pb is absorbed from the gut, transported in the bloodstream, and deposited in tissues such as the liver, kidney, and bone. The Pb behavior in this study is discussed in three parts: (1) lead shot erosion in the ventriculus, (2) the change of lead concentrations in blood samples (blood lead level; BLL) over time, and (3) the Pb distribution in tissues and excretion in feces.

3.1.1. Lead shot erosion in the ventriculus

Lead shots were retained in the ventriculuses of three of the four ducks, and during the 29 days of feeding, 17%–100% of the Pb shots were lost from their ventriculuses (Supplementary Table S4), showing the ratios of loss differed among individuals. The amount of shot found in the ventriculus is a strong predictor of the Pb concentration in liver and bone tissues(Mateo et al., 2001), therefore, erosion in the ventriculus is the first step in Pb absorption, so the different ratios of Pb loss are likely to have a significant impact on the subsequent behavior of Pb: transportation in the bloodstream, deposition in tissues, and the resultant toxic effects. In fact, the maximum BLL (C_{max}) and Pb concentration in all bone samples (described below) increased as the shots loss ratio increased, although not proportionately.

The disappearance rate tended to be higher in individuals with many fragments of non-Pb shot in the ventriculus (Supplementary Table S5). This tendency suggests these fragments played an important role in the erosion of Pb shots. The ducks were fed only dry food and not supplied with additional grit, so other hard objects did not enter the ventriculus and this may have slowed shots erosion. Therefore, the possibility that Pb shots would erode more quickly in the wild cannot be ruled out.

Gastric acid pH may also influence Pb shot erosion. Gastric acid pH differs among avian species, for example the pH is 2.2 in mallard ducks (*Anas platyrhynchos*), 1.3 in bald eagles (*Haliaetus leucocephalus*), and 3.7 in chickens (*Gallus gallus domesticus*; (Beasley et al., 2015). This may explain the differences in Pb damage in different species exposed to the same amount of Pb shots. Thus, we should bear in mind that the result of this study may not pertain to other avian species.

3.1.2. The change in BLL over time

The BLL are shown in Figure 1. Detail of the severity levels, assessed according to the interpretation in Anseriformes(Franson and Pain, 2011), are shown in Supplementary Figure S1 and Supplementary Table S6. There were no BLL differences between the samples collected before exposure and the control group. Among the Pb-exposed birds, BLL ranged from 0.26–1.26 mg/L one day after Pb exposure, reaching a sub-clinical poisoning level in three ducks and a severe clinical poisoning level in the other duck. The C_{max} of each duck was 0.65 mg/L, 0.93 mg/L, 2.40 mg/L, and 3.74 mg/L; a clinical level was reached in two ducks and a severe clinical level in the other two ducks. Throughout the 29 days feeding, BLLs remained above the sub-clinical poisoning level in all Pb-exposed ducks. At 28 days after exposure, BLLs ranged from 0.40–2.95 mg/L, with one duck at a severe clinical poisoning level, one duck at a clinical poisoning level, and the remaining two at a sub-clinical level. These levels were 18.6%–78.8% of the C_{max}. BLL stayed above the clinical poisoning level for at least nine days in all exposed ducks, two of which were above the severe clinical poisoning level for over ten days.

C_{max} was reached at almost simultaneously among the Pb-exposed ducks; at 10 or 14 days after exposure, despite the different amounts of Pb shots retained in their ventriculuses. This may be mainly due to a change in speed of Pb-shot erosion and Pb transportation to tissues, because BLL represents the circulating levels of Pb released from the shots and Pb accumulating in tissues. As the shots lose volume, their surface areas are reduced and, thus, the speed of erosion would be reduced. However, the speed of Pb transportation would also decrease as Pb accumulates in tissues. It is assumed that as the amount of Pb transported decreased, the amount of Pb eroded in the ventriculus was reflected in the BLL.

3.1.3. The Pb distribution in tissues and excretion in feces

The Pb concentrations in the tissue and bone samples and the severity levels are shown in Table 1 (dry weight) and Supplementary Table S7 (wet weight), and are visualized in Supplementary Figure S2-S3. The highest accumulation was observed in the bones, followed by the cecum, kidneys, jejunum, and liver. There were no significant differences between the edge of the bone and the center for the femur and

tibiotarsus. Except for the humerus, Pb levels in all bones reached a clinical or sub-clinical level. All Pb levels in kidneys were under or at the sub-clinical level. The Pb concentration in the liver of one duck reached a clinical level, while the other three ducks' livers had normal Pb levels.

Lead concentration in fecal samples varied widely, even among samples taken on the same day (Supplementary Table S8). Fecal Pb concentration is shown in Supplementary Figure S4 with a stacked line graph, revealing that Pb was detected throughout the 29-day feeding period.

Surprisingly, the cecum and jejunum had as high Pb accumulation as the bones, kidney, and liver, which are well-known Pb accumulation tissues. This suggests that these intestinal tracts are important for Pb absorption in Muscovy ducks, which may be due to their well-developed intestinal tracts; the Muscovy duck is omnivorous and has a relatively long cecum (Clench, 1999).

Bones are also thought to be an important aspect of Pb distribution. When comparing the ducks in this study with swans found dead due to Pb poisoning (Figure 2; Ishii et al., 2018), the Pb accumulation in the bones of the ducks was as high as that in the bones of the swans, but Pb accumulation was lower in the ducks' kidneys and livers. One explanation may be the assumption that the swans died before the full amount of Pb accumulated in their bones, although it should be noted that the context of swans' exposure is unknown. Alternatively, this finding may suggest that a larger proportion of the Pb was transferred to the bones rather than to other tissues in the Muscovy ducks, possibly alleviating the toxicity. However, bone is known to have a long Pb half-life(Fisher et al., 2006; Pain et al., 2019), suggesting that there is a risk of prolonged Pb retention in the body, creating a Pb carrier and source of Pb exposure for predators.

Bones composed of trabecular tissue and containing bone marrow accumulate Pb rapidly (Ishii et al., 2018). The high Pb accumulation in the bones during our study may be a result of the ducks being young and hence their bones being were not aerated and still had abundant blood flow. In fact, high Pb bone levels in teal ducklings are reported in field studies, where active bone formation has been considered the cause (Mateo et al., 2001). This idea is also supported by the small differences in Pb concentration between the edge and center of bones.

311 Shots retained in the ventriculus and high Pb accumulation in bones suggests
312 that Pb could remain in the body and possibly affect ducks' health beyond 30 days
313 after exposure; It is also known that Pb stored in bone is released back into the blood
314 through the bone remodeling (Pounds, 1991). A study with a longer feeding period
315 would provide a greater understanding of the effects of low-dose exposure.
316
317 **3.2. The toxic effects of low-dose Pb exposure**
318 During the 29-day feeding period, no causalities or other serious clinical
319 symptoms such as lethargy, wing drooping, or neurological signs were observed. All
320 ducks showed good appetites and increased their body weights, except for one Pb-
321 exposed duck, which showed mild anorexia and lethargy 21 days after exposure
322 (Supplementary Table S9). There were no remarkable differences in either blood
323 biochemical analysis or metallothionein content between the Pb-exposed and control
324 groups (Supplementary Figures S5–S6, Supplementary Tables S10–15). In the
325 pathological examination, Pb specific lesions were not observed (Torimoto et al., n.d.).
326 Based on these results, it appears that Pb damage in the Muscovy ducks was
327 at a sub-clinical or lower level. In contrast, some Pb concentrations in the blood and
328 bones were above the clinical level. This disparity might be a result of the feeding
329 environment; the ducks in this study had permanent access to food and were in good
330 nutritional status. This is also supported by the fact that there was little effect on the
331 body weights in this experiment, despite the known association between Pb and poor
332 body condition in waterfowl(De Francisco et al., 2003; Franson and Pain, 2011;
333 Rodríguez et al., 2010). Foraging in the wild is more difficult and nutrient deficiencies
334 occur easily, even more so if Pb exposure causes adverse effects to birds' health.
335 Therefore, ducks in the wild might suffer relatively more severe Pb damage.
336 On the other hand, disparity between Pb concentrations and clinical
337 symptoms have been reported in field studies; In a study at Argentina hunting hotspot,
338 only three ducks with BLL of 0.65, 0.73, and 5.75 mg/kg showed clinical signs, even
339 though more than five ducks' BLLs had reached the clinical poisoning levels(Ferreyra
340 et al., 2015). It is difficult to estimate the severity of clinical symptoms based on
341 concentrations in specific organs alone; it may be necessary to combine concentrations
342 in multiple organs.

Ht and δ-ALAD activity tended to decrease in the Pb-exposed group (Figure 3, Supplementary Tables S16–17). δ-ALAD activity markedly decreased one day after exposure, to 4.8%–38.3% of the activity measured before Pb exposure. The δ-ALAD activity remained below 15% of the activity measured before Pb exposure until 28 days after exposure. Although no significant external anemic symptoms were observed, the δ-ALAD activity in the blood decreased acutely in response to Pb exposure, indicating signs of anemia. This was supported by the low Ht. If low δ-ALAD activity is sustained for longer periods, for example 39 days, which is the lifespan of duck erythrocytes (Brace and Altland, 1956), anemic symptoms may be observed. Furthermore, there is a possibility that δ-ALAD binds to Pb, causing an immediate rise in the BLL. A study on humans found that ALAD-2, one isoform of ALAD, appears to bind lead in a less toxic form (Kelada et al., 2001). Thus, the reduced δ-ALAD activity observed during this study suggests a decrease in resistance to Pb. If this hypothesis is true, Muscovy ducks may experience greater damage when re-exposed to Pb because δ-ALAD decreases after the first exposure. Further studies are needed to assess the risk of reduced δ-ALAD activity.

For each immune and oxidative stress gene, the relative mRNA expression levels are shown in Supplementary Table S18 and Supplementary Figure S7, and are exhibited in a heat map in Figure 4. The expression of TLR3 in the liver was significantly higher in the Pb-exposed group than in the control group (p = 0.043, Wilcoxon test). The PCA and Spearman's correlation tests suggested TLR3 expression was correlated with Pb concentration (Supplementary Figure S8, Supplementary Table S19). TLR3 is a key receptor for sensing the double-stranded RNA that is generated during virus replication (Son et al., 2015; Thompson et al., 2011). Several studies have shown that TLR3 is involved in viral infection, clearance, and pathogenesis in mammals (Goffic et al., 2006; Hutchens et al., 2008; Leung et al., 2014). In geese, TLR3 plays an important role in recognizing viruses (Yong et al., 2018). The increased expression of TLR3 observed in this study indicates that Pb enhanced the immunity of the ducks, but this result is important in the sense that it indicated that the immune system was disrupted. There is a possibility that the disturbance in TLR3 contributes

to a decline in the survival rate of wild birds. The result in this study is consistent with a previous finding showing increased TLR3 expression as a result of Pb exposure in the bursa of Fabricius of the Japanese quail, but the mechanism remains unclear (Nain and Smits, 2011). Nickel ions can directly ligate to and trigger toll-like receptor 4 on human dendritic cells (Rachmawati et al., 2013), and a similar trend has been reported for gold and TLR3 (Rachmawati et al., 2015). Although further study is needed to elucidate these interactions, there is a possibility that Pb directly ligates to TLR3 and disrupts the immune system.

The α-diversity, which represents microbial variation in a single sample, is reflected by the Chao-1 index and Shannon index (Supplementary Figure S9). Neither index showed a significant difference between the groups nor was there a significant change over time in the Pb-exposed group ($p > 0.1$, Wilcoxon test). Further, there were no significant relationships between either index and the daily BLL ($p > 0.1$, Spearman's test). These results suggest that α-diversity was not influenced by Pb exposure in Muscovy ducks, in contrast to previous studies (Eggers et al., 2019; Kou et al., 2019). However, it should be taken into consideration that our ducks were young and still growing, and this might have influenced the α-diversity.

The relative abundances of dominant microbiota phyla and families are illustrated in Supplementary Figure S10. The OTUs that showed a significant correlation with BLL are described in Table 2 ($p < 0.05$, Spearman's test). Eighteen of the 39 significant OTUs were in the order *Clostridiales*; 11 were in the family *Lachnospiraceae* and five were in *Ruminococcaceae*. Nine of the *Lachnospiraceae* and all *Ruminococcaceae* OTUs were positively correlated to BLL, suggesting that the *Lachnospiraceae* and *Ruminococcaceae* families were most relevant Among the OTUs. *Lachnospiraceae* and *Ruminococcaceae* have been suggested to have cholesterol-reducing properties (Antharam et al., 2016) and are associated with blood lipid levels in humans (Fu et al., 2015). Research suggests that Pb affects mouse metabolism through a change in the microbiota (Gao et al., 2017; Xia et al., 2018), and our results provided similar indications for Muscovy ducks.

It has been reported that the microbiota differs along the intestine in chickens, particularly in the cecum, and influences its functions (Xiao et al., 2017). While we

did not examine cecal feces, we found a high accumulation of Pb in the cecum, which seemed to contribute to Pb absorption. Clearer results may be obtained from sampling cecal feces.

During this study, prolonged shot retention in the ventriculuses and high Pb accumulation in the bones were observed. Faster erosion of Pb shots in the ventriculus led to a faster BLL increase, resulting in more serious symptoms. However, if the erosion is slow, Pb shots will be retained in the body for longer, and ducks will be chronically affected by Pb. Furthermore, because the symptoms are relatively mild, the ducks may become Pb carriers. Similarly, for Pb accumulations in bones, the Pb may be retained for long periods and accumulate during an animal's lifetime (Pain et al., 2019), resulting in chronic Pb exposure. A Pb carrier could act as a source of Pb exposure for predators and could also lead to the spread of unexpected infectious diseases due to the effect on immune systems.

The limitations of this study must be mentioned. Firstly, the sample size was relatively small. Considering animal welfare, the number of individuals was reduced to the minimum possible for statistical analysis. For the same reason, only males were used instead of females, which might be more affected by hormones. Secondly, there were large differences between individuals in this study, particularly with regard to the amount of Pb erosion in the ventriculuses. Although this observation of individual variation in Pb shot erosion was an important result, it did alter the net amount of Pb absorbed in the bodies of each duck, which made it undeniably difficult to interpret the results. Further research designed with an understanding of these limitations is needed to capture the actual situation more accurately.

4. Conclusions

During this study, we evaluated the erosion of Pb shots in the ventriculus, the change of Pb concentration over time, and the Pb accumulation in the tissues caused by low-dose oral exposure to Pb shots. Our results suggested that low-dose Pb would remain in the body and possibly affect health for more than 30 days after exposure. Judging from feeding status, blood biochemical analysis, and pathological

examination, damage due to Pb exposure was sub-clinical or lower in the Muscovy ducks in this study. In contrast, some Pb concentrations in the blood and bones were above the clinical level. Furthermore, there was potential sub-clinical damage to the ducks, as signs of anemia were observed through δ-ALAD activity and Ht. Gene expression analysis suggested there was an immune system disturbance. Changes in the microbiota suggested there was a disturbance in lipid metabolism. These potential effects may lead to an increase in mortality risk in the wild, which should not be ignored.

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FIGURES and TABLES

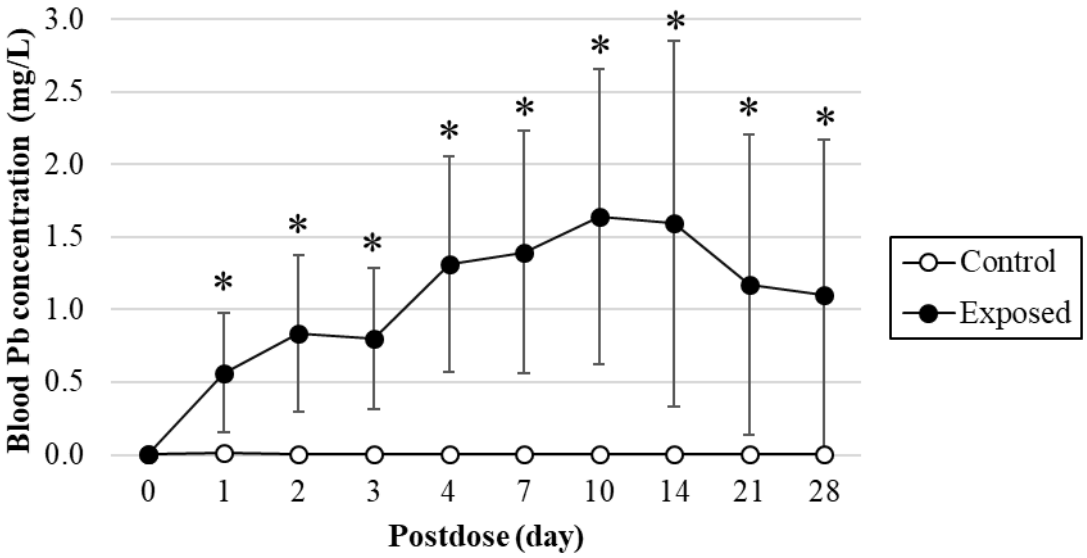

Fig 1

Changes in mean blood lead (Pb) concentration of control and Pb-exposed Muscovy ducks. The horizontal axis indicates the number of days after exposure. The vertical axis indicates blood Pb concentration (mg/L). Data points are means ± SD. Filled circle markers indicate the Pb-exposed group, and open markers indicate the control group. * indicates a significant difference between the Pb-exposed and control groups ($p < 0.05$, Wilcoxon test).

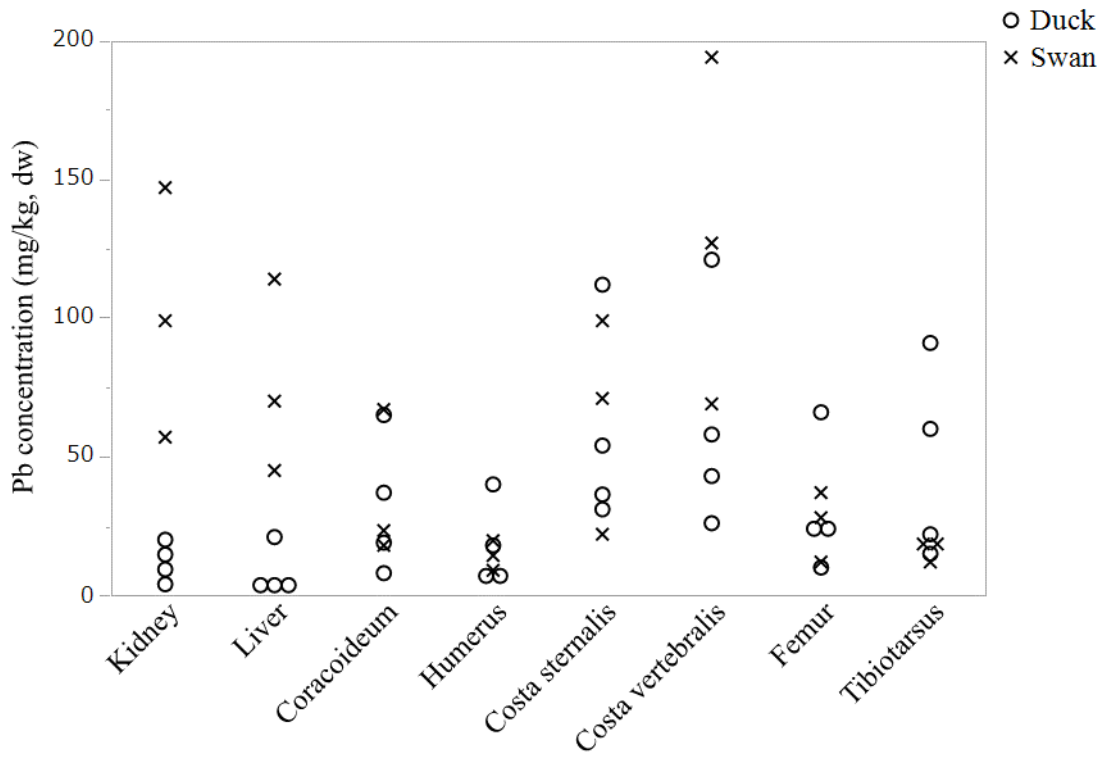

Fig 2

Pb concentrations (mg/kg, dry weight) in the liver, kidney (cranial lobes), and bones of Pb-exposed Muscovy ducks from this study compared with concentrations from Pb-poisoned swans found dead in a field (Ishii et al., 2018). Open circles indicate the Pb concentrations of Pb-exposed Muscovy ducks. Cross marks indicate the Pb concentrations from the poisoned swans.

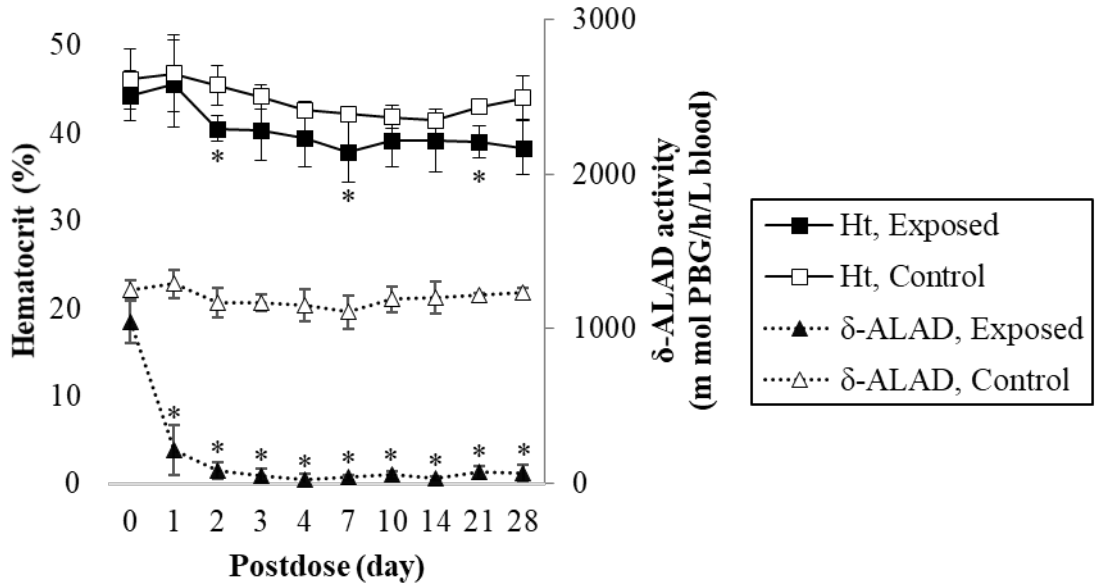

Fig 3

Changes in mean hematocrit (Ht) and δ-aminolevulinic acid dehydratase (δ-ALAD) activity in control and Pb-exposed Muscovy ducks. The horizontal axis indicates the number of days after exposure. The left vertical axis indicates Ht (%). The right vertical axis indicates δ-ALAD activity (m mol PBG/h/L blood). Solid lines and square markers indicate Ht. Dashed lines and triangle markers indicate δ-ALAD activity. Filled markers indicate the Pb-exposed group and open markers indicate the control group. Data points are means ± SD. * indicates a significant difference between the Pb-exposed and control groups ($p < 0.05$, Wilcoxon test).

PBG: porphobilinogen, product of δ-ALAD

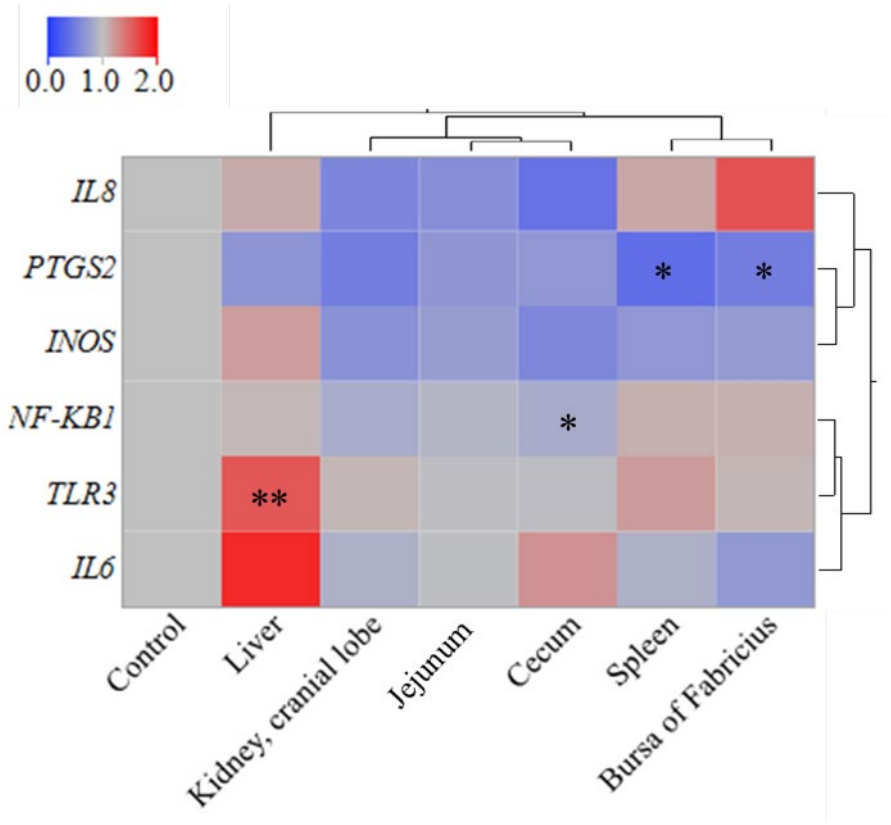

Fig 4

Relative expression of immune functional genes and oxidative stress genes (the copy number of the target gene divided by the copy number of beta-actin). The relative mRNA expression of interleukin-6 (IL6), interleukin-8 (IL8), inducible nitric oxide synthase (INOS), nuclear factor-kappa B subunit 1 (NF-KB1), prostaglandin-endoperoxide synthase 2 (PTGS2), toll-like receptor 3 (TLR3) in each tissue are shown using a heat map. Data are expressed as mean fold change versus the control group. * indicates $p < 0.1$, and ** indicates $p < 0.05$.

Table 1. Pb concentrations in each tissue of control and Pb-exposed Muscovy ducks (mg/kg, dry weight) and the number of Pb-exposed ducks per severity level.

Tissue	Control group (N=6)		Exposed group (N=4)		Severity level (Franson and Pain, 2011)		
	Mean	Range	Mean	Range	Sub-clinical poisoning	Clinical poisoning	Severe clinical poisoning
Costa sternalis	0.10	0.09 - 0.14	61.7	26.1 - 120.6	N=0	N=4	-
Costa vertebralis	0.09	0.06 - 0.13	57.7	30.5 - 111.9	N=0	N=4	-
Femur, edge	0.12	0.08 - 0.19	48.0	11.0 - 95.2	N=1	N=3	-
Tibiotarsus	0.07	0.06 - 0.09	46.9	14.6 - 91.1	N=1	N=3	-
Tibiotarsus, edge	0.12	0.07 - 0.25	40.1	14.4 - 77.9	N=1	N=3	-
Coracoideum	0.10	0.05 - 0.26	32.3	8.0 - 65.3	N=2	N=2	-
Femur	0.07	0.05 - 0.09	31.1	10.5 - 65.8	N=1	N=3	-
Cecum	0.04	0.02 - 0.08	20.3	0.9 - 71.3			
Humerus	0.05	0.04 - 0.06	17.9	6.4 - 39.9	N=1	N=1	-
Kidney, caudal lobe	0.03	0.02 - 0.04	13.1	6.6 - 29.4	N=1	N=0	N=0
Kidney, middle lobe	0.05	0.03 - 0.08	13.0	6.0 - 27.5	N=2	N=0	N=0
Kidney, cranial lobe	0.04	0.03 - 0.06	10.4	3.9 - 19.2	N=3	N=0	N=0
Jejunum	0.03	0.01 - 0.06	8.4	0.8 - 27.4			
Liver	0.02	0.01 - 0.02	8.0	3.5 - 21.0	N=0	N=1	N=0
Pancreas	0.02	0.02 - 0.03	6.3	2.4 - 15.9			
Lung	0.03	0.02 - 0.04	4.0	1.2 - 11.9			
Spleen	0.03	0.02 - 0.04	3.6	1.1 - 10.1			

Cerebellum	0.05	0.01 - 0.10	3.5	1.8 - 5.2
Bone marrow	0.02	0.01 - 0.05	2.5	0.46 - 5.0
Cerebrum	0.03	0.01 - 0.05	2.0	0.71 - 4.2
Midbrain	0.10	0.01 - 0.31	2.0	1.1 - 3.7
Adrenal gland	0.05	0.01 - 0.12	1.5	0.68 - 3.4
Genital	0.02	0.01 - 0.03	1.0	0.48 - 2.3
Thyroid	0.06	0.01 - 0.14	0.91	0.45 - 1.8
Rectum	0.04	0.02 - 0.10	0.82	0.21 - 1.7
Bursa of Fabricius	0.03	0.02 - 0.06	0.74	0.23 - 1.7
Bile	0.01	0.005 - 0.02	0.69	0.15 - 1.9
Heart	0.01	0.01 - 0.02	0.34	0.15 - 0.81
Pectoralis muscle	0.02	0.01 - 0.04	0.16	0.08 - 0.35
Supracoracoideus muscle	0.01	0.01 - 0.02	0.09	0.04 - 0.21
Fat	0.01	0.003 - 0.04	0.05	0.02 - 0.11

Note: Tissues are arranged in descending order of mean Pb concentration of the exposure group. In the “Severity level (Franson and Pain, 2011)” column, blank cells and dashes indicate that toxicity levels for the tissue of the Pb exposed birds did not fall within the category. Bursa of Fabricius: N = 5, bile: N = 4 in the control group.

Table 2. List of bacterial operational taxonomic units (OTUs) significantly associated with blood lead concentration ($p < 0.05$).

Phylum	Class	Order	Family	Genus	OTUs	r_s	p value
Actinobacteria	Actinobacteria_c	Actinomycetales	Actinomycetacea	Actinomyces	__	0.61	0.004
	Coriobacteriia	Coriobacteriales	Coriobacteriaceae	PAC001041_g	PAC001041_s	0.51	0.018
Bacteroidetes	Bacteroidia	Bacteroidales	AC160630_f	__	__	0.45	0.040
			Bacteroidaceae	Bacteroides	Bacteroides plebeius	0.52	0.015
			Prevotellaceae	PAC001422_g	PAC001422_s	0.45	0.041
				Prevotella	JRNP_s	0.45	0.040
					Prevotella corporis	0.62	0.003
			RF16_f	EU009805_g	EU009805_s	0.45	0.040
Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus	Enterococcus cecorum	0.53	0.014
			Leuconostocaceae	Leuconostoc	__	0.45	0.038
			Streptococcaceae	Lactococcus	Lactococcus lactis	0.73	0.000
	Clostridia	Clostridiales	Lachnospiraceae	Anaerotignum	Anaerotignum lactatifermentans	0.63	0.002
				Blautia	Blautia obeum	-0.47	0.031
				Clostridium_g24	PAC001164_s	0.44	0.045

	Frisingicoccus	Coprococcus catus	-0.47	0.031
	GU302778_g	__	0.53	0.013
	PAC000692_g	__	0.55	0.009
	PAC001043_g	PAC001043_s	0.54	0.012
		PAC001449_s	0.46	0.038
	PAC001138_g	PAC001305_s	0.46	0.036
	PAC001283_g	PAC001283_s	0.56	0.009
	PAC002196_g	PAC002196_s	0.45	0..41
Mogibacterium	PAC001236_s	PAC001644_s	0.44	0.047
Peptostreptococcaceae	Intestinibacter	Intestinibacter bartlettii	0.50	0.022
Ruminococcaceae	Fournierella	__	0.55	0.009
	PAC001663_g	PAC001663_s	0.50	0.022
	Sporobacter	PAC001055_s	0.52	0.015
		PAC001306_s	0.46	0.036
	Subdoligranulum	DQ455909_s	0.46	0.037

	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	__	__	0.55	0.010
				CCMM_g	__	-0.50	0.022
				Merdibacter	Merdibacter massiliensis	0.58	0.006
	Negativicutes	Veillonellales	Veillonellaceae	Dialister	PAC001039_s	0.46	0.038
Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	LARJ_g	FJ683333_s	0.51	0.020
					GQ898126_s	0.49	0.025
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonas	__	-0.45	0.039
	Epsilonproteobacteria	Campylobacterales	Campylobacteraceae	Campylobacter	EU470464_s	0.52	0.016
	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	__	-0.56	0.008
Tenericutes	Mollicutes	Acholeplasmatales	Acholeplasmataceae	FJ507320_g	FJ507320_s	0.51	0.018

Note: r_s indicates Spearman correlation coefficient.