The Journal of Veterinary Medical Science

NOTE

Wildlife Science

Relationship between blood test values and blood lead (Pb) levels in Black-headed gull (*Chroicocephalus ridibundus*: Laridae)

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J. Vet. Med. Sci.
82(8): 1124–1129, 2020
doi: 10.1292/jvms.20-0246

Received: 26 April 2020
Accepted: 7 June 2020
Advanced Epub:
19 June 2020

ABSTRACT. Few studies have evaluated immunosuppression due to lead accumulation below the overt toxicity threshold. If low levels of lead accumulation cause immunosuppression in birds, those birds could become more susceptible to pathogens. We aimed to determine if low levels of lead accumulation lead to immunosuppression in Black-headed gulls (*Chroicocephalus ridibundus*). Gulls were captured in Tokyo-bay and Mikawa-bay from January to April 2019. Their blood samples were analyzed for eight items. The data were analyzed to evaluate the correlation between lead concentrations and the variables from each bay. Lead was positively correlated with the percentage of heterophils and heterophil and lymphocyte ratio and negatively with lymphocytes. Thus, low lead accumulation levels may induce changes in percentage of the heterophils and lymphocyte.

KEY WORDS: blood status, *Chroicocephalus ridibundus*, immunosuppression, Japan, Lead accumulation

Lead is a common pollutant found in various avian species [10, 31, 32]. When birds are acutely poisoned with high lead levels, they develop lethargy, or death without clinical signs. In the chronic phase, they develop various clinical signs, such as aplastic anemia, thin eggshell, immunosuppression, and neurological symptoms (e.g., depression and torticollis) [13, 27, 30, 51, 52, 64]. Lead accumulation affects the survival of some avian species [33, 39, 45, 50], such as Common pochard (*Aythya ferina*) and California condor (*Gymnogyps californianus*) [22, 27, 47, 63].

Lead has mainly become an environmental pollutant because of its use in bullets and fishing sinkers [54, 56, 57]. Some overseas regions have banned or restricted the use of lead bullets to prevent environmental pollution and reduce their impact on wildlife [34, 57, 58]. In Japan, the use of lead bullets has only been restricted in Hokkaido since 2000 [4, 37]. Nakata *et al.* [41] reported that environmental lead pollution had mainly occurred in southern areas of Japan by assessing lead accumulation levels in wild caught two Rattus species (*Rattus norvegicus* and *Rattus rattus*). In Japan, some wild birds are affected by lead pollution, such as gooses and swans [40, 42–44]. If the birds inhabiting Japan become an immunosuppression due to a lead accumulate, they may become more susceptible to pathogens, such as highly pathogenic avian influenza virus (HPAIV).

Susceptibility (immunity) can be evaluated by calculating the ratio of heterophils and lymphocytes (H/L ratio) [21]. Heterophils and lymphocytes account for 95% of all leukocytes in avian peripheral blood [12]. Heterophils and lymphocytes are measured for avian health assessment because heterophils are involved in coping with immediate infections and lymphocytes being more consequential in communicable diseases though cellular and humoral immunity [2, 15, 31, 52]. A higher percentage of lymphocytes than heterophils reflects proper immune function in some avian species [29, 48].

Black-headed gull (*Chroicocephalus ridibundus*) is a migratory bird that are affected by lead accumulation [28, 36, 38, 46]. The gulls migrate to Japan from Russia, North China, and Mongolia [3, 5, 37], and in winter, they live in loosely knit locks [1, 19]. Although the gull is a familiar species, its population declined by approximately 45% in the European region from 1989 to 2014 [49]. There is concern about mass mortality due to oil spills or chemical accumulation [18, 36]. Additionally, they are highly susceptible to HPAIV [25, 35, 60], so if lead accumulation causes an immunosuppression regardless its accumulated level, various

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Table 1. Sex and age classification of Black-headed gulls (*Chroicocephalus ridibundus*) captured from January 2019 to April 2019 at two bays

Area (Bay)	Male/Adult	Female/Adult	Male/Yealing[a)]	Male/Yearling[a)]
Tokyo-bay	36	30	2	0
Mikawa-bay	5	16	2	2

a) Yearling data was not available for statistical analysis due to the small sample size.

risks may increase—such as spreading and deaths from these viruses. Therefore, we conducted this study to assess if low levels of lead accumulation cause immunosuppression by analyzing the relationship between lead accumulation level and blood parameters in the Black-headed gull.

This study was approved by the University's Laboratory Animal Ethics Committee (approval number: 30S-47), Ministry of the Environment, Chiba prefecture (approval number: 739-1568, 1667), and Aichi prefecture (approval number: 588-5). The study was conducted from January 2019 to April 2019 in the Ichikawa City, Chiba Prefecture, Japan (Tokyo-bay) and Gamagori City, Aichi prefecture, Japan (Mikawa-bay). The Black-headed gulls were captured by noose trap or whoosh net [9, 55]. Gaunt *et al.* [17] reported that ethical blood sampling requires that the collection be less than 1% of their body mass. Therefore, the captured gulls were weighed quickly, and blood of less than 0.5% of the body mass was collected from the right median metatarsal vein or posterior branch of the brachial vein using a heparinized syringe and 26G needle. After hemostasis, we placed a metal ring on its right tarsus, and released it. We evaluated the following indexes for health condition and body condition: runny nose and wheezing by inspection and auscultation; feces evaluation for green stool, bloody stool, and diarrhea; checked the keel score for nutritional status [17]; dehydration was confirmed by palpation of the conjunctiva, and a skin pinch test; assessing neurological symptoms such as torticollis, nystagmus, and dizziness by observing the captured gull's behavior.

We assessed age because lead accumulation levels are closely related to a bird's age [24, 47]. Age was evaluated using their plumage [3]; gulls that had a juvenile plumage were classified as "Yearling", and did not have a juvenile plumage were "Adult" [20]. Sex was identified using polymerase chain reaction (PCR) [7, 61]. Red blood cell (RBC; 10^6cells/μl) and leukocyte (WBC; cells/μl) were counted using a Neubauer hemocytometer after staining with Natt and Herrick's solution [8, 53]. Packed cell volume (PCV; %) was determined using the microhematocrit method [53]. Hemoglobin (Hb; g/d*l*) was measured as an absorbance at 540 nm (Synerger HTX, BioTek®, Chicago, IL, USA) [11]. Percentage of heterophil (Het) and lymphocyte (Lym) were evaluated using blood smears that were stained with Light-Giemsa staining solution and H/L ratio was calculated. A blood clot was used to measure the lead levels in gull's peripheral blood. Briefly, blood samples which was autoclaved beforehand were digested with 5 m*l* of 30% nitric acid (Kanto Chemical Corp., Tokyo, Japan) and 1 m*l* of 30% hydrogen peroxide (Kanto Chemical Corporation) in a microwave digestion system (Berghof, Eningen, Germany). Lead concentration were measured with an inductively coupled plasma–mass spectrometer (Agilent Technology, Tokyo, Japan) [59]. Analytical quality control was performed using the certified reference material of Seronorm™ Trace Elements Whole Blood L-2 (Sero, Billingstad, Norway). Replicate analysis of reference material showed good accuracy (relative standard deviation less than 3%) and recoveries (95–105%). Finally, the measurements were converted to the amount of lead per μl of clots (Lead).

Statistical analyses were performed with software R (ver. 3.5.0) and Stata (ver. 14.0). A Wilcoxon rank-sum test was performed to determine whether age (Yearling and Adult), sex, and area (Tokyo-bay and Mikawa-bay) were associated with the lead level. If a significant difference was found in the three indices, a Spearman's rank correlation was performed between the hematologic parameters and lead concentration for each index. For all analyses, $P<0.05$ was considered statistically significant.

A total of 93 birds (68 from Tokyo-bay, 25 from Mikawa-bay) were captured (Table 1). The sample size of the Yearling was small; therefore, only the Adult was included in the statistical analysis. Lead concentrations were significantly higher in the Tokyo-bay than in the Mikawa-bay, but there were no significant differences in lead concentrations between male and female gulls. Therefore, only area was included in the Spearman's rank correlation. The hematologic parameters from each bay are shown in Table 2. In the Tokyo-bay population, Het was significantly positively correlated with Lead ($P<0.05$; $\rho= 0.6$; Fig. 1a), and Lym was significantly negatively correlated with Lead ($P<0.05$; $\rho= -0.6$; Fig. 1b). Same trend was observed in the Mikawa-bay population ($P<0.05$; $\rho= 0.6$; Fig. 2a; and $P<0.05$; $\rho= -0.5$; Fig. 2b, respectively). Further, the H/L ratio in the gulls from each bay was significantly positively correlated with Lead ($P<0.05$; Tokyo-bay: $\rho= 0.5$, Mikawa-bay: $\rho= 0.6$; Fig. 3). The other hematologic parameters were not significantly correlated with lead concentration ($P>0.05$).

The lead levels of the gull population in each bay were classified as low-level accumulation based on previous report [62]. Although little is known about the transitions (changes) in blood concentrations following low lead accumulation, high lead accumulation has been reported in some avian species. For instance, some seagull species quickly develop many symptoms, such as anemia and neurological symptoms, following exposure to high lead concentrations [23]. On the other hand, regarding clinical symptoms related to low lead accumulation, Hollady *et al.* [23] reported that domestic pigeons (Columbidae) exposed to low lead accumulation did not exhibit any clinical signs. Therefore, we concluded that gulls were more likely to have been a steady, low lead accumulation since the beginning of exposure, than exposed to high and gradually decreasing from that levels.

Lead pollution in gulls in each bay was recognized as short-term exposure for the following two reasons. First, studies have reported that lead in the avian body is excreted into the eggshell [6], therefore, it is possible that males can be exposed to a higher level of lead accumulation than females, especially during the season after breeding. However, we did not observe any differences in lead levels

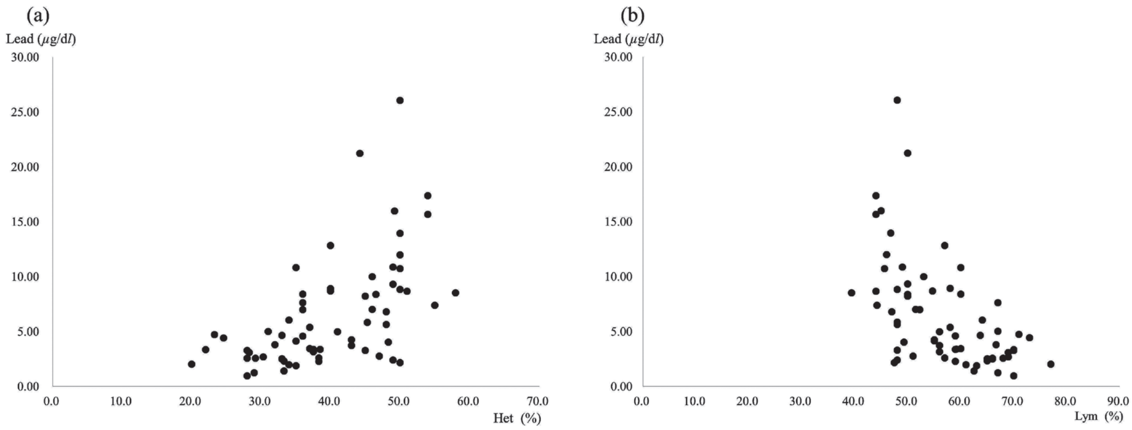

Fig. 1. Relationship between heterophils (Het; a) or lymphocytes (Lym; b) and lead concentration (Lead) in the Tokyo-bay population. The scatter diagram indicates the proportion of Het (a), and Lym (b) and is presented on the horizontal axis (unit: %). The vertical axis corresponds to the lead concentration in each diagram. In Tokyo-bay, 36 male Adults and 30 female Adults were captured from January 2019 to April 2019. Het was significantly positively correlated with lead concentration ($P<0.05$; $\rho= 0.6$), and Lym was significantly negatively correlated with lead concentration ($P<0.05$; $\rho= -0.6$).

Table 2. The values of 10 blood status items in Black-headed gulls (*Chroicocephalus ridibundus*) from two bays

a. Tokyo-bay

Item (unit)	N[a)]	Mid[b)]	Max[c)]	Min[d)]
RBC[e)] ($*10^6$cell/μl)	66	3.02	3.81	1.52
Hb[f)] (g/dl)	66	8.86	19.66	4.13
PCV[g)] (%)	66	39.0	55.0	30.0
WBC[h)] (cell/μl)	66	9,200	13,000	2,100
H/L ratio	66	0.65	1.47	0.26
Het[i)] (%)	66	38.4	58.0	20.0
Lym[j)] (%)	66	56.5	77.0	39.4
Mon[k)] (%)	66	2.0	6.3	0.2
Eos[l)] (%)	66	0.7	3.0	0.0
Bas[m)] (%)	66	0.0	1.0	0.0
Lead[n)] (μg/dl)	66	4.68	26.05	0.94

b. Mikawa-bay

Item (unit)	N	Mid	Max	Min
RBC ($*10^6$cell/μl)	21	2.06	3.23	1.76
Hb (g/dl)	21	17.50	24.39	9.56
PCV (%)	21	40.0	44.0	30.0
WBC (cell/μl)	21	4,400	9,600	2,800
H/L ratio	21	0.53	1.34	0.22
Het (%)	21	33.7	57.0	17.7
Lym (%)	21	64.0	79.0	42.5
Mon (%)	21	1.7	3.6	0.3
Eos (%)	21	0.0	2.5	0.0
Bas (%)	21	0.0	1.0	0.0
Lead (μg/dl)	21	2.73	5.92	1.50

a) Sample size, b) median value, c) maximum value, d) minimum value, e) red blood cell number, f) hemoglobin g) packed cell volume, h) white blood cell number i) percentage of heterophil, j) percentage of lymphocyte, k) percentage of monocyte, l) percentage of eosinophil m) percentage of basophil n) lead concentration.

by sex during the winter season, thus, we concluded that the gull populations were accumulated in a short time period. Second, bird banding has shown that Black-headed gulls migrate across the two bays in one day [Ushine unpublished]. Although the gulls in each bay were able to interact, there were significant differences in blood lead levels between each population. It was possible that the gulls were exposed to lead in the short term at each bay.

Although most of them are high level accumulation, there are some theories that explain the effect of lead accumulation on immunity,

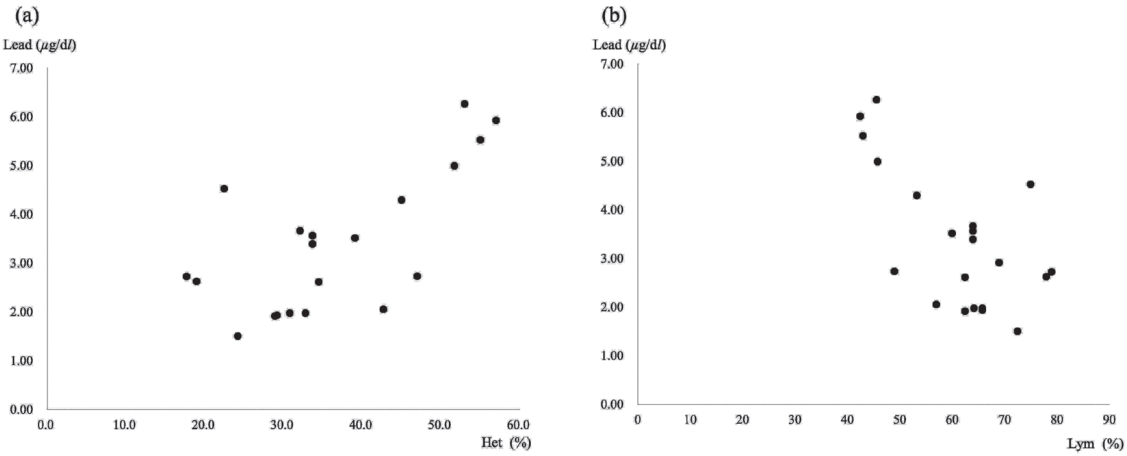

Fig. 2. Relationship between heterophils (Het; a) or lymphocytes (Lym; b) and lead concentration in the Mikawa-bay population. The scatter diagram indicates the proportion of Het (a), and Lym (b) and is presented on the horizontal axis (unit: %). The vertical axis corresponds to the lead concentration (Lead) in each diagram (unit: μg/d*l*). In Mikawa-bay, five male Adults and 16 female Adults were captured from January 2019 to April 2019. Het was significantly positively correlated with lead concentration ($P<0.05$; $\rho = 0.6$), and Lym was significantly negatively correlated with lead concentration ($P<0.05$; $\rho = -0.5$).

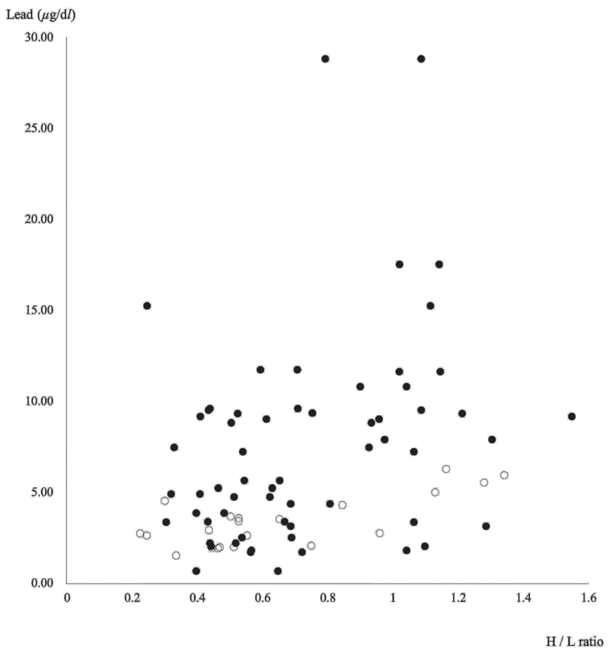

Fig. 3. Relationship between the heterophil and lymphocyte ratio (H/L ratio) and lead concentration (Lead) in each bay. The scatter diagram indicates the proportion of the H/L ratio on the horizontal axis. The vertical axis corresponds to the lead concentration in each diagram (unit: μg/d*l*). In Mikawa-bay (open circle), five male Adults and 16 female Adults were captured from January 2019 to April 2019. In Tokyo-bay (filled circle), 36 male Adults and 30 female Adults were captured from January 2019 to April 2019. H/L ratio was significantly positively correlated with lead concentration ($P<0.05$; Tokyo-bay: $\rho = 0.5$, Mikawa-bay: $\rho = 0.6$).

including the suppression of lymphocyte number [14, 16, 40]. In this study, the leukocyte did not change with Lead; however, there were changes in the differential blood counts. That is, lead caused to decrease the percentage of Lymphocyte and increase Heterophil, which was possibly a foreign body reaction or an inflammatory response that were targeted to lead. Increased inflammatory responses cause to the production of cytokines [26], which suppress immune function. Therefore, we hypothesized that lead accumulation has a direct immunosuppressive effect due to the decrease in lymphocytes and an indirect effect due to the increase in heterophils.

The limitations of the study were the small sample size in each bay, especially of Yearlings. Analysis stratified by age was considered important for assessing environmental pollutants that accumulate in the short and long term in the avian body. Additionally, this study was designed to be as non-invasive as possible. Using blood samples rather than necropsy precluded the kind of detailed analysis that could be performed when measuring parameters such as the lead concentration in organs.

ACKNOWLEDGMENTS. We thank all the staff of the Gyotoku Bird Observatory Society NPO. We are grateful to Ms. S. Hirota, Ms. H. Sasaki, Ms. H. Otsubo, Dr. S. Moriguchi, and Dr. T. Kato from the Department of Wildlife medicine of the Nippon veterinary and life science University of Tokyo prefecture for the great cooperation in this survey. We also thank to Mr. K. Uchida and Dr. K. Tokita for many advises. Finally, we are grateful to Ms. N. Hirano amd Mr. T. Ichise for the critical help with lead measurement. This work was supported by the Sasakawa Scientific Research Grant from The Japan Science Society and the Environment Research and Technology Development Fund (SII-1) of the Environmental Restoration and Conservation Agency of Japan. This research was also supported by JST/JICA, SATREPS (Science and Technology Research Partnership for Sustainable Development; No. JPMJSA1501) and Program for supporting introduction of the new sharing system (JPMXS0420100619).

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