Study on the effects of migration and lead pollution on host immunity, and infection of influenza A virus in Black-Headed Gull (*Chroicocephalus ridibundus*)

Summary of Doctoral Thesis

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Approximately 20% of avian species have migratory ecology worldwide. Migration requires a large amount of energy; thus, it is considered that migration has a trade-off with physiological functions such as immunity. It is presumed that the immune function of migratory birds is suppressed by the supply of energy, and their infection rate is increased by immunosuppression during the migration period. However, there are no previous reports on this hypothesis.

Regarding this hypothesis, we reviewed previous reports on influenza A, which is thought to be carried by migratory birds. Laridae and Anseriformes groups are reported to have high mortality due to influenza A infection. The species of these avian groups have differences in terms of energy indices (body condition) such as body mass, muscle mass, and fat mass, associated with their migration. However, it has not been clarified how the changes in body condition associated with the migration affect to their infection of pathogens. In particular, any knowledge about influenza A and body condition is poor in Laridae compared to Anseriformes.

It is known that infection of pathogens is affected not only by the decreased body condition but also by exposure to environmental pollutants. Among environmental pollutants, lead (Pb), which is a heavy metal, has been reported to show biological effects in many wild avian species. The population of California condor (*Gymnogyps californianus*) declined sharply from 1982 to 1986 due to the feeding of Pb-contaminated meat. The number of condors on the earth is currently only approximately 1000. The white-headed duck (*Oxyura leucocephala*) is also on the verge of extinction due to Pb pollution. However, previous reports on the actual state of Pb pollution in migratory birds, and immune effects in migratory birds, are limited to some species. Therefore, this study targeted Black-headed gulls (*Chroicocephalus ridibundus*) that overwinter on the main island of Japan. I have been doing research on this species about their migration ecology since undergraduate. The purpose of this study was to evaluate the effects on immunity in Black-headed gulls due to changes in their body condition during wintering and Pb contamination, and to clarify the relationship with influenza A infection.

Chapter 1. Elucidating the effects of body condition and lead (Pb) contamination on immunity in Black-headed gulls during the migration period

From 2018 to 2021, a total of 326 Black-headed gulls were captured from Tokyo-bay and Mikawa-bay. These gulls showed significant differences in blood Pb levels between autumn migration, spring migration, and wintering during the winter season (when wintering was used as a reference, statistic was -0.59 and 95% confidence interval [95%

CI] was -0.76 to -0.42 in the autumn migration, and statistic was -0.26 and 95% CI was -0.42 to -0.10 in the spring migration). Considering the results and the characteristics of Pb pharmacokinetics, it was suggested that Black-headed gulls had been contaminated with Pb in Japan.

Focusing on the forage resources as the Pb contamination source for Black-headed gulls, stable isotope ratio analysis of nitrogen (δ15N) and carbon (δ13C) was performed using the primary covert by estimating the foraging during breeding season. The range of δ15N was 10.00 to 17.00 ‰ and δ13C was -28.00 to -12.00 ‰. Considering previous reports, it was possible that the gulls of the study population were contaminated by these benthic organisms, such as marine crustaceans and shellfish in the study area.

Correlation analysis was performed between immune parameters (number of white blood cells, proportion of heterophils and lymphocytes, ratio of heterophils and lymphocytes (H/L ratio), mRNA levels of CD4 and CD8α), and blood Pb levels using the gull's peripheral blood. The Pb level had a significant relationship with the proportion of heterophils (correlation coefficient, ρ= 0.22) and the proportion of lymphocytes (ρ= -0.22), and it was observed that Pb levels had a negative correlation with the gull's immune function. In addition, Pb levels in 4.0 μg/dL peripheral blood have been shown to affect the proportion of heterophils and lymphocytes, which was lower than the standard Pb level of 20 μg/dL peripheral blood that causes an immune effect in birds as per the previous reports.

Body condition is defined as the body condition index (BCI), which is the first principal component analysis of the body weight, muscle mass, and fat mass of the captured Black-headed gulls. Correlation analysis between BCI and immune parameters showed that the BCI was positively correlated with the number of white blood cells (ρ= 0.33) and the proportion of lymphocytes (ρ= 0.22). In contrast, the proportion of heterophils (ρ= -0.21) and H/L ratio (ρ= 0.22) showed a negative correlation with BCI.

Finally, multiple regression analysis and sequential logistic regression analysis showed that the gull's immune function was affected by Pb level and body condition change depending on migration in the winter season. The proportion of heterophils and lymphocytes were significantly positive, and negatively affected by the Pb levels in all winter season, including autumn migration, wintering, and spring migration (proportion of heterophil, statistic was 7.49, and 95% CI was 3.50 – 11.49 in autumn migration, statistic was 14.49, 95% CI 8.92 – 20.05 in wintering, and statistic was 0.71, and 95% CI was 0.38 – 1.05; proportion of lymphocytes, statistic was -7.32, and 95% CI was -11.43 to -3.21 in autumn migration, statistic was -14.26, and 95% CI was -19.93 to -8.59, and statistic was -0.77, and 95% CI was -0.09 to -0.46 in spring migration). In particular,

during spring migration, BCI had a significant effect on the proportion of heterophils (statistic was 4.17, and 95% CI was -5.61 to -2.72) and lymphocyte (statistic was 3.89, and 95% CI was 2.54 to 5.23). The spring migration had a higher Pb level than the autumn migration (P value was < 0.01). In a previous report, the body mass and muscle mass were the lowest in the spring migration during winter season. Therefore, it was considered that gull's immunity was affected by body condition and Pb pollution in the spring migration by the energy trade-off between migration and immune function.

Chapter 2. How does body condition and Pb pollution affect influenza A infection in a Black-headed gull?

The antibody against influenza A virus in gull plasma was used to evaluate the relationship among body condition, Pb pollution, and influenza A infection. By modifying the protocol of the commercially available ELISA kit, the ELISA results were defined as history of infection, and the absorbance in plasma obtained by ELISA was defined as the amount of antibody. Analysis of history of infection and amount of antibody during the autumn migration, wintering, and spring migration (defined in Chapter 1). As a results of the history of infection and amount of antibody were significantly high during the spring migration (P value was <0.05).

Next, I analyzed whether the gull's body condition and Pb pollution affect the history of infection and amount of antibody. Body condition and Pb level showed a significant difference in the history of infection (statistic was -0.31and 95% CI was -0.60 to -0.02 in body condition, and the statistic was 0.98 and 95% CI was 0.15 – 1.81 in Pb level). It was confirmed that the gulls which had decreased their BCI was occurred to high population of history of infection, and the which had increased their Pb level was occurred to high population of history of infection. In the antibody positive group, the relationship between the amount of antibody, BCI, and blood Pb level was evaluated using multiple regression analysis. BCI was not significantly related to the amount of antibody (statistic was 0.03, P value was 0.21, and 95% CI was -0.01 to 0.06), while the higher Pb levels significantly decreased the amount of antibody (statistic was -0.08, P value was 0.03, and 95% CI was -0.16 to -0.01).

Since blood Pb levels and BCI were significantly related to the history of infection, if gulls' group which had low BCI and high blood Pb level had more the history of infection than gulls' group which had the other states. Gulls' BCI was divided two group, high BCI and low BCI using the result of first principal component in Chapter 1, and gulls' blood Pb level was also dived two group, Pb high and Pb low using the discussion about the effect proportion of immune cell on Pb level, 4.0 μg/dL in peripheral blood. As a result

of the analysis, the group with a low BCI and high Pb had significantly more history of infection than the group with a high BCI and a low Pb (P value was 0.01). In addition, it was confirmed the amount of antibody was affect by high/low Pb in each period. It was significantly low amount of antibody was found in high Pb only in spring migration (P= 0.04).

Finally, we examined the effects of body condition and blood Pb level on the history of infection and the amount of antibody in the winter season. As a result of sequential logistic analysis using the amount of antibody and the history of infection as the objective variables, and the BCI and blood Pb concentration as the explanatory variables, no significant relationship was found between all the variables during the autumn migration. In the wintering, blood Pb level had a significant positive effect on the history of infection (statistic was 0.18 and 95% CI was 0.03 – 0.33), and during the spring migration, the history of infection had a significant effect on BCI (statistic was -0.42 and 95% CI was -7.99 to -0.43) and Pb level (statistic was 2.16 and 95% CI was 0.03 – 4.28), and the amount of antibody had a significant effect on the BCI (statistic was 0.20, P value was 0.047, and 95% CI was -0.34 to -0.00).

Based on these survey results, the infection to influenza A differs between autumn and spring in terms of the degree of influence from Pb levels and BCI. Both Pb level and BCI affected the amount of antibody, and the history of infection in spring migration. Considering that the blood Pb level and BCI were significantly decreased during the spring migration compared to the wintering, it has been suggested that the significant increase in influenza A antibody levels and history of infection was also due to the ecological factor that Black-headed gulls shared habitat with other species of birds such as ducks and Anseriformes.

Through this study, some basic findings were clarified regarding the effects of changes in body condition due to migration and environmental Pb pollution on the immune function or influenza A infection in Black-headed gulls. Among the findings elucidated or suggested in this study, the following three points have important implications for the management of wildlife infectious diseases: 1. Immunity was maintained by increasing body conditions and suppressed by increasing Pb levels. 2. Black-headed gulls' heterophil and lymphocyte proportion were affected by the Pb level 4.0 μg/dL in peripheral blood, and 3. Influenza A infection was affected by gulls' migratory periods, body condition, and Pb levels.