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The effects of migration on the immunity of Black-Headed Gulls (*Chroicocephalus ridibundus*: Laridae)

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ABSTRACT. In order to elucidate the relationship between migration period and immunity related to susceptibility, we conducted research on Black-headed gulls (*Chroicocephalus ridibundus*). We captured 260 gulls and collected their peripheral blood. Their leukocyte (WBC) count, percentages of heterophils (Het) and lymphocytes (Lym), heterophil and lymphocyte ratio (H/L ratio), and CD4 and CD8α expression levels (CD4 and CD8α, respectively) were quantitatively analyzed over three migration periods (Autumn migration, Wintering, Spring migration). In Adult gulls, WBC counts and CD4 levels significantly increased. Moreover, the Het and H/L ratio decreased from the Autumn migration to Wintering. Conversely, only WBC counts and CD4 levels measurements significantly decreased from Wintering to Spring migration ($P<0.05$). The tested parameters of the Tokyo-bay population show a greater significant difference than the measurements of immunity of the Mikawa-bay population. This study suggests that the migratory period has a negative effect on an aspect of the immune system. Including the period-difference in the immune systems in the local population, it is necessary to investigate the relationship between the ecology of migratory birds and their immunity.

KEY WORDS: CD4, CD8α, *Chroicocephalus ridibundus*, immunological measurements, migration period

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Migratory birds carry some infectious pathogens over a wide area [6, 23]. Moreover, their own migratory behavior has a negative impact on their body condition [32, 46]. Therefore, the possibility of carrying pathogens is closely related to their immunity [32].

As in mammals, avian host susceptibility is associated with their immunity [25, 29, 36]. Heterophils and lymphocytes account for 95% of all leukocytes in avian peripheral blood [7]. Heterophils are responsible for the initial response to pathogens by signaling from Toll-like receptors and pathogen elimination and work as innate immunity to protect oneself from viral infection [12, 15, 18, 20]. Lymphocytes eliminate specific pathogens by cell-mediated or humoral immunity [16].

In the peripheral blood, CD4 protein is expressed on the surface of the immune cells, which work on maintaining immune function (e.g., monocytes and helper T lymphocytes) [22]. CD8 protein is expressed on the surface of immune cells, which work on eliminating pathogens (e.g., killer T cells and natural killer cells) [40, 41]. In humans and mice, the expression ratio of CD4 to CD8 proteins is used to diagnose several viral, autoimmune, and infectious diseases that infect immune cells [1, 23].

Similarly, avian immunity is measured by the ratio of heterophils to lymphocytes (H/L ratio) [24, 39]. In avian species with higher percentages of lymphocytes than of heterophils, the H/L ratio is used to evaluate immune function [8, 17]. The immune systems of these species appropriately maintain lymphocytes at a higher number than that of heterophils [21, 31]. Avian CD4 and CD8 expression levels are measured through mRNA or protein levels using polymerase chain reaction (PCR) and antigen-antibody interactions, respectively [13, 35]. PCR targets the sequences of CD4 and CD8 mRNAs. However, this method also needs to

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take species differences into account, as nucleotide sequences of CD4 and CD8 mRNAs are species-dependent [35]. Therefore, quantitatively evaluating CD4 and CD8 mRNAs is difficult without a common avian primer designed against a common sequence across avian species. To date, species-specific CD4 or CD8 primers are only available for ducks and chickens. We aim to clarify the relationship between the migratory behavior of birds and host immunity. We targeted Black-headed gulls in this study as these gulls had been reported to have various zoonotic diseases, such as Salmonella, antimicrobial-resistant *Escherichia coli*, and highly pathogenic avian influenza. Moreover, the gulls were easier to capture than other wild migratory species [3, 10, 16, 21, 26, 30, 34, 37].

MATERIALS AND METHODS

Sampling and identification of sex and age

Black-headed gulls were caught in Tokyo-bay (Ichikawa City, Chiba Prefecture, Japan) and Mikawa-bay (Gamagori City, Aichi Prefecture, Japan) from January 2019 to March 2020. Their migration period was divided into three periods: "Autumn migration", the period of increased numbers of gulls in these areas; "Spring migration", the period of decreased numbers of gulls to no gulls in these areas; and "Wintering", the period between these two previously mentioned periods when the population did not change significantly [44]. Gulls were captured using a noose trap or a whoosh net. Blood collection of less than 1% of the body mass is the standard in avian ethics [14]. Thus, a captured gull was immediately placed inside a cloth bag, weighed, and a blood volume of less than 0.5% of the body mass was collected from a right median metatarsal vein or posterior branch of the brachial vein using a heparinized 26-G injection needle and syringe.

After confirming hemostasis, the captured gull was released with a metal ring from the Ministry of the Environment placed on the gull's right tarsus [31]. This study was approved by the laboratory animal ethics committee of Nippon Veterinary and Life Science University (approved number: 30S-47), Ministry of the Environment, Chiba Prefecture (approved number: 1667, 2020), and Aichi Prefecture (approved number: 31-588-5). Gulls' ages were estimated and classified into two groups: under 1-year-old (Yearling) or over (Adult), according to plumage [4]. Sex was determined by a molecular biology method, using collected blood [43].

Evaluating hematological parameter using leukocytes

Leukocytes (WBC) were counted using a Neubauer hemocytometer (cells/μl) after staining with Natt and Herrick's solution [5, 38]. Percentages of heterophils (Het), lymphocytes (Lym), and H/L ratio were calculated by a blood smear stained with Wright–Giemsa solution.

Designing PCR primers of CD4 and CD8α mRNA

Degenerate primers for CD4 and CD8 mRNAs (Table 1) were designed using the database (CD4: Supplementary Table 1, CD8α: Supplementary Table 2) at the National Center for Biotechnology Information (NCBI). The primer for CD8 mRNA was designed with reference to CD8α. The PCR products were obtained by the degenerated PCR with a gull cDNA and KOD Fx Neo DNA polymerase (Toyobo, Osaka, Japan: Table 2). Avian-common CD4 and CD8α primers (Table 3) were designed using the amplifier, which had the highest homology in the sequence of the PCR products amplified by the degenerated PCR for several avian species.

In the sequences obtained by PCR using avian-common CD4 and CD8a primers (Table 4), unique CD4 and CD8α sequences were selected, which did not match any registered avian sequence, and the gull's specific CD4 and CD8α primers were designed (species-specific CD4 and CD8α primers; Table 5). These primers were tested by PCR at each annealing temperature using KOD Fx Neo (Table 4). Each PCR product was sequenced to confirm the target sequence amplification (accession number: LC533369, LC533370).

Preparation for gene expression analysis

The collected blood was centrifuged at 12,000 × g for 5 min to separate blood cells and plasma. RNA was extracted (Trizol Reagent, Thermo Fisher Scientific, Waltham, MA, USA) from 50 or 100 μl of homogenized blood cell components that were homogenized before coagulation. cDNA was synthesized from extracted RNA following manufacturers' protocol from ReverTra

Table 1. Sequences and Tm values of degenerated primers for CD4 and CD8α used in this study

Target	Primer name	Forward / Reverse	Primer sequence (5′-3′)	Tm value (unit: °C)
CD4	CD4F0518	Forward	GTTTCTTGTCTYGTSCAT	48.8
	CD4R0518	Reverse	TTCTGCASCTGGGTCTGA	44.0
CD8α	CD8F0518	Forward	GTSTCCTGCACTGCTCCT	52.0
	CD8R0518	Reverse	CTGCATCKTCGTCTTCTGGT	49.0

Table 2. PCR protocols for degenerate primers

3-step cycle	Cycle condition	Number of cycles
Pre-denaturation	94°C, 2 min	1
Denaturation	98°C, 10 sec	
Annealing	Tm –5°C, 30 sec	30 cycles
Extension	68°C, 10 sec	
Final-extension	72°C, 5 min	1

When Tm value was different between forward and reverse primer, the Tm value was obtained by –5°C from the average temperature of forward and reverse.

Ace qPCR RT kit (Toyobo) or cDNA Synthesis kit (Toyobo). gDNA was removed with Recombinant DNase I (Takara Bio, Kusatsu, Japan). No PCR amplicons were detected from the RNA samples treated with DNase I.

Preparation of real-time PCR

TA cloning was performed using PCR-amplified products from species-specific CD4 and CD8α primers. PCR-amplified products were ligated into a pTAC2 vector and transformed into competent cells (carbenicillin-resistant *E. coli*). The plasmid was recovered from *E. coli* using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI, USA), and the plasmid sequence was analyzed using an ABI PRISM 3100 Genetic Analyzer (Thermo Fisher Scientific). Plasmid DNA with the confirmed cloned target regions was used to calibrate real-time PCR using KOD SYBR qPCR mix (Toyobo). The amplified copy number was logarithmically converted and evaluated as expression levels of CD4 and CD8α mRNA (CD4 and CD8α, respectively) per 1 μl of blood lysate.

Statistical analysis

We set four indices as independent variables: migration periods, area, sex, and age [45]. The three migration periods were categorized as follows: Autumn migration, Wintering, and Spring migration. Age was categorized as 0 for Yearling and 1 for Adult. Immunological measurements (WBC, Het, Lym, H/L ratio, CD4, CD8α) were set as dependent variables. First, normal distribution was confirmed in all variables by the Shapiro-Wilk test. Then, an *F*-test was performed to consider the multicollinearity of these independent variables, and variables without significant relationships were used for model analysis. We performed statistical analysis for each independent variable item that had a difference in the dependent variables. The general linear model was used if all variables showed normal distribution; otherwise, the generalized linear model was used for evaluation.

To evaluate changes in immunological items during the migration period, two of three periods were selected and analyzed. The significance level was set to 0.05. All analyses were performed using R (ver. 3.5.0) or Stata (ver. 14.0).

RESULTS

A total of 260 gulls were captured in two areas from January 2019 to March 2020. In Tokyo-bay, 81 male Adults, 78 female Adults, three male Yearlings, and a female Yearling, and in Mikawa-bay, 32 male Adults, 53 female Adults, five male Yearlings, and seven female Yearlings. The results of immunological measurements are shown in Tables 6 and 7. Yearlings were not analyzed

Table 3. Sequences and Tm values of avian-common primers for CD4 and CD8α used in this study

Target	Primer name	Forward / Reverse	Sequence (5′-3′)	Tm value (°C)
CD4	CD4F0803	Forward	AGTCAGTATCTCACTGCATGTC	58.4
	CD4R0803	Reverse	CTTTGATGTGAAGGTATTAGGTTTTCAG	64.7
CD8α	CD8αF0803	Forward	TGGAACCCTTCATTTCATCGTCTTCAT	71.5
	CD8αR0803	Reverse	GAAGGCAGGCTGGCTGGGGCTGAAGTACAG	80.8

Table 4. PCR protocols for avian-common and species-specific primers

3-step cycle	Cycle condition	Number of cycles
Pre-denaturation	94°C, 2 min	1
Denaturation	98°C, 10 sec	
Annealing	Tm –5°C, 30 sec	35 cycles
Extension	68°C, 10 sec	
Final-extension	72°C, 5 min	1

When Tm value was different between forward and reverse primer, the Tm value was obtained by –5°C from the average temperature of forward and reverse

Table 5. Sequences and Tm values of species-specific primers for CD4 and CD8α used in this study

Target	Primer name	Forward / Reverse	Sequence (5′-3′)	Tm value (°C)
CD4	L.ridibundusCD4F	Forward	TTGAATGTGAGTGATTAGAGGGCTTTTAT	52.1
	L.ridibundusCD4R	Reverse	GATTAATCAATGGAGAATCTGCATGGACAT	53.7
CD8α	Specific0924CD8αF	Forward	TTTTGGAACCCTTCATTTCATCGTCTTC	53.2
	Specific0924CD8αR	Reverse	AAGGCAGGCTGGCTGGGGCTGAAGTAG	62.0

Table 6. Results of the immunological test items (WBC, Het, Lym) in each migration period

		Autumn migration		Wintering		Spring migration	
		Adult	Yearling	Adult	Yearling	Adult	Yearling
WBC							
Tokyo	Male	5,200 (8,000–2,400)	-	10,400 (13,000–5,200)	10,200 (11,800–8,600)	4,600 (9,200–2,400)	3,200
		18	0	51	2	14	1
	Female	6,000 (8,000–2,400)	8,000	10,000 (12,200–6,000)	-	5,600 (12,200–3,200)	-
		15	1	41	0	23	0
Mikawa	Male	5,700 (8,000–4,000)	-	7,200 (10,400–4,000)	6,000 (9,800–4,400)	4,000 (5,200–3,000)	6,400 (9,600–3,200)
		4	0	20	3	5	2
	Female	3,600 (6,400–2,400)	-	6,200 (10,400–4,000)	7,400 (8,800–6,000)	6,400 (10,000–3,400)	6,800 (8,400–5,600)
		12	0	20	2	23	5
Het							
Tokyo	Male	39.1 (61.8–26.4)	-	36.0 (51.0–20.0)	36.8 (51.0–22.5)	45.9 (64.5–27.3)	27.2
		18	0	51	2	14	1
	Female	41.8 (50.0–19.9)	28.6	35.0 (58.0–22.0)	-	41.8 (55.5–27.0)	-
		15	1	41	0	23	0
Mikawa	Male	38.2 (41.8–31.8)	-	45.7 (57.0–23.6)	34.0 (52.7–23.0)	38.9 (47.9–29.9)	27.5 (33.3–21.7)
		4	0	20	3	5	2
	Female	43.6 (58.2–33.6)	-	45.7 (57.0–23.6)	29.1 (34.5–23.6)	39.1 (64.5–17.7)	34.0 (52.7–23.0)
		12	0	20	2	23	5
Lym							
Tokyo	Male	59.0 (71.8–35.5)	-	61.0 (77.0–44.0)	59.0 (74.0–44.0)	50.0 (72.0–34.5)	67.0
		18	0	51	2	14	1
	Female	56.4 (80.0–49.0)	68.0	61.0 (75.0–39.4)	-	57.3 (70.0–43.6)	-
		15	1	41	0	23	0
Mikawa	Male	60.9 (67.3–54.5)	-	54.0 (75.5–41.8)	63.8 (77.0–46.4)	60.4 (70.0–51.8)	70.5 (77.0–64.0)
		4	0	20	3	5	2
	Female	55.0 (64.5–40.9)	-	60.5 (75.5–41.8)	70.5 (75.5–65.5)	60.5 (79.0–34.5)	65.5 (77.0–46.4)
		12	0	20	2	23	5

Tokyo: Tokyo-bay, Mikawa: Mikawa-bay. Upper row was indicated a median value, middle row was a maximum value–minimum value in parentheses, and lower row was sample size. The hyphen indicates that there was no test value because there was no sample. Leukocyte number, WBC (cell/μl); percentage of heterophil, Het (%); percetage of lymphocyte, Lym (%).

due to their small sample size. The adult immunological measurements had no significant difference in regards to sex, but had a significant difference in some measurements in areas (WBC, CD4, and CD8α). In the Shapiro-Wilk test, no independent variables had normal distributions (z>0.05); therefore, these immunological items were analyzed using a generalized linear model with mixed sex, for each area (Tables 8 and 9).

In the Tokyo-bay population, all items excepting the H/L ratio showed significant changes between Autumn migration and Wintering. These coefficient values (WBC, Lym, CD4, and CD8α) were significantly increased, and Het significantly decreased (P<0.05) from the Autumn migration to Wintering. Conversely, there was no significant change in Het and Lym in the Mikawa-bay population.

In the Tokyo-bay population, the opposite changes that occurred over the Autumn migration to Wintering were observed in the Wintering to Spring migration, whereas in Mikawa-bay population, this change only occurred in CD4. In each bay, WBC and CD8α significantly increased only from Wintering to Spring migration (P<0.05). In Autumn and Spring migration, the Lym significantly decreased only in the Tokyo-bay population during Spring migration, and CD4 significantly increased in the Mikawa-bay population during Spring migration.

DISCUSSION

In the Tokyo-bay population, there were significant differences in the tested parameters between the migration and non-migration period; however, for the Mikawa-bay population, leukocyte counts and CD8α mRNA levels were different between the non-migration period and either migration period. Moreover, only the CD4 expression levels were significantly different between both migration periods and the non-migration period. The reason for not observing similar seasonal changes in the Tokyo-bay and Mikawa-bay populations could be due to differences in the sample size between the two populations.

In the Tokyo-bay population, the percentage of lymphocytes decreased during the migration periods. This result is supported by previous reports that analyzed migratory birds and suggested that this trend of decreasing lymphocytes during migration was caused by stress and energy trade-off with migratory behavior [32, 33, 46]. Northern wheatears (*Oenanthe oenanthe*) prioritize

Table 7. Results of the immunological test items (H/L ratio, CD4, CD8α) in each migration period

		Autumn migration		Wintering		Spring migration	
		Adult	Yearling	Adult	Yearling	Adult	Yearling
H/L ratio							
Tokyo	Male	0.67 (1.74–0.37)	-	0.62 (1.16–0.26)	0.73 (1.16–0.30)	0.90 (1.87–0.38)	0.41
		18	0	51	2	14	1
	Female	0.74 (1.02–0.24)	0.42	0.56 (1.47–0.31)	-	0.73 (1.27–0.39)	-
		15	1	41	0	23	0
Mikawa	Male	0.63 (0.77–0.47)	-	0.85 (1.36–0.31)	0.53 (1.14–0.30)	0.64 (0.92–0.43)	0.40 (0.52–0.28)
		4	0	20	3	5	2
	Female	0.79 (1.42–0.52)	-	0.64 (1.36–0.61)	0.42 (0.53–0.31)	0.65 (1.87–0.22)	0.49 (0.69–0.28)
		12	0	20	2	23	5
CD4							
Tokyo	Male	4.08 (6.24–3.97)	-	5.25 (5.73–2.38)	5.19 (5.26–5.12)	4.39 (4.78–2.96)	4.69
		18	0	46	2	14	1
	Female	4.09 (4.43–3.95)	3.95	5.11 (5.77–3.29)	-	4.52 (5.85–2.18)	-
		15	1	39	0	23	0
Mikawa	Male	3.16 (3.18–3.15)	-	5.08 (5.55–2.90)	5.08 (5.13–4.78)	3.81 (4.03–3.80)	4.95 (4.96–4.95)
		4	0	19	3	5	2
	Female	3.14 (5.04–2.64)	-	5.08 (5.55–2.90)	4.97 (5.27–4.68)	4.08 (5.28–3.68)	5.08 (5.27–4.68)
		12	0	19	2	23	5
CD8α							
Tokyo	Male	1.61 (2.29–1.17)	-	2.32 (3.63–1.85)	2.31 (2.35–2.26)	1.99 (2.60–1.88)	1.99
		18	0	46	2	14	1
	Female	1.48 (2.03–0.31)	0.31	2.25 (2.65–1.49)	-	2.10 (2.74–1.59)	-
		15	1	39	0	23	0
Mikawa	Male	1.25 (1.49–0.65)	-	2.02 (2.82–0.75)	2.06 (2.19–1.70)	1.96 (2.53–1.72)	1.52 (1.63–1.39)
		4	0	19	3	5	2
	Female	1.14 (1.91–0.24)	-	2.02 (2.82–0.75)	1.78 (2.06–1.50)	1.84 (2.72–1.51)	2.06 (2.19–1.50)
		12	0	19	2	23	5

Tokyo: Tokyo-bay, Mikawa: Mikawa-bay. Upper row was indicated a median value, middle row was a maximum value, minimum value in parentheses, and lower row was sample size. The hyphen indicates that there was no test value because there was no sample. Rates of percentage of heterophil and lymphocyte, H/L ratio; number of CD4 mRNA, CD4 {log (copy/μl)}; number of CD8α mRNA, CD8α {log (copy/μl)}.

Table 8. Results of generalized linear model analysis between migration periods of the Black-headed gulls (*Chroicocephalus ridibundus*)

Dependent variables	Independent variables	Autumn migration and Wintering (n=122)	Wintering and Spring migration (n=127)	Autumn migration and Spring migration (n=69)
WBC	Coefficient	**7,162.64**	**–4,435.18**	**821.20**
	P-value	**<0.0001**	**<0.0001**	**0.001**
H/L ratio	Coefficient	–0.07	**0.18**	0.053
	P-value	0.21	**0.001**	0.171
Het	Coefficient	**–4.71**	**6.28**	1.99
	P-value	**0.01**	**<0.0001**	0.08
Lym	Coefficient	**6.64**	**–6.34**	**–2.28**
	P-value	**<0.0001**	**<0.0001**	**0.041**
CD4	Coefficient	**8.73**	**–5.58**	1.08
	P-value	**<0.0001**	**<0.0001**	0.10
CD8α	Coefficient	**5.87**	**–1.32**	**2.64**
	P-value	**<0.0001**	**0.016**	**0.0001**

Model analysis was performed to select two of the three migration periods in Tokyo-bay. N was indicated sample number. Leukocyte number, WBC (cell/μl); percentage of heterophil, Het (%); percetage of lymphocyte, Lym (%); rates of percentage of heterophil and lymphocyte, H/L ratio; number of CD4 mRNA, CD4 {log (copy/μl)}; number of CD8α mRNA, CD8α {log (copy/μl)}. Values in bold correspond to significant differences (P<0.05).

Table 9. Results of generalized linear model analysis between migration periods of the Black-headed gulls (*Chroicocephalus ridibundus*)

Dependent variables	Independent variables	Autumn migration and Wintering (n=57)	Wintering and Spring migration (n=69)	Autumn migration and Spring migration (n=44)
WBC	Coefficient	**1,836.59**	311.32	**889.29**
	P-value	**0.002**	0.59	**<0.0001**
H/L ratio	Coefficient	–0.02	–0.07	–0.05
	P-value	0.84	0.47	0.42
Het	Coefficient	–2.66	–2.71	–2.21
	P-value	0.33	0.33	0.19
Lym	Coefficient	3.56	2.55	2.20
	P-value	0.21	0.35	0.18
CD4	Coefficient	**10.98**	**–4.50**	**4.87**
	P-value	**0.004**	**0.006**	**<0.0001**
CD8α	Coefficient	**7.54**	1.69	**4.11**
	P-value	**0.016**	0.16	**<0.0001**

Model analysis was performed to select two of the three migration periods in Mikawa-bay. N was indecated sample number. Leukocyte number, WBC (cell/μl); percentage of heterophil, Het (%); percentage of lymphocyte, Lym (%); rates of percentage of heterophil and lymphocyte, H/L ratio; number of CD4 mRNA, CD4 {log (copy/μl)}; number of CD8α mRNA, CD8α {log (copy/μl)}. Values in bold correspond to significant differences (P<0.05).

energy storage for migration and suppress immune functions, making it difficult to control oxidative stress and inflammatory responses [11]. In Black-headed gulls, body mass and pectoral muscles are reduced during migration [44]. In avian species, the pectoral muscle is the largest muscle and produces myokines, which are involved in inducing cytokine and chemokine production [9, 42]. No relationship has been reported between myokines and avian migration [46]; therefore, it is necessary to elucidate the relationship between avian migration ecology and such cytokines.

No sex differences in migration periods were found in this study. Male gulls arrive earlier than females at breeding sites [19]. Male American redstarts (*Setophaga ruticilla*), Eurasian skylarks (*Alauda arvensis*), and Asian short-toed larks (*Calandrella cheleensis*) also migrate earlier than females and have been shown to already produce some reproductive-related hormones at Spring migration, influencing their body mass and immune function [41, 47]. In this study, some tested parameters were significantly different between the Spring migration period and other periods; therefore, it is necessary to evaluate sex-related differences with a sufficient sample size.

The functions and mechanisms of action of CD4 and CD8α in wild migratory species have not yet been elucidated. The antibody-antigen reaction is performed by flow cytometry with monoclonal antibodies (mAb) against CD4 and CD8 [2, 6, 10, 27]. However, the accuracy of these measurements is questionable as there may be different protein structures between different avian species, and thus, one particular mAb may not work for all species [11].CD4 and CD8α proteins are expressed on the membrane surface of dendritic cells and granulocytes, such as monocytes, which play an important role in innate immunity [15, 36]. Considering the results of the mRNA levels of CD4 and CD8α, while excluding the CD8α data of the Mikawa-bay population, it can be considered that the migration ecology negatively affects the expression levels of CD4 and CD8α.

The limitations of this study were that the sample sizes were not uniform between each migration period as we targeted field populations. It is necessary to investigate these regional differences with a sufficient sample size and clarify whether there are differences in the immunity of the same species due to regional differences. Moreover, Yearlings were not analyzed in this study. CD8α protein levels increase quantitatively and qualitatively with age in chickens [28]; further studies with larger samples are needed to analyze this relationship in wild bird species.

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