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Novel host factors associated with resistance to highly pathogenic avian influenza in wild birds inferred from primary cell culture

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Bird species differ in the sensitivity to the highly pathogenic avian influenza virus (HPAIV). Here, we infected fibroblasts from 11 bird species with the H5N1 HPAIV strain A/chicken/Yamaguchi/7/2004. These species were categorized into three groups based on previous studies: HPAI-resistant (rock pigeon, hooded crane, white-necked crane, and Japanese crane), HPAI- susceptible (chicken, mountain hawk-eagle, northern goshawk, peregrine falcon, and golden eagle), and those with unknown susceptibility to HPAI (Okinawa rail and Japanese white stork). We performed gene expression analysis to identify genes uniquely upregulated in the HPAI-resistant species and determine genetic markers of HPAIV susceptibility. We found that two genes involved in antiviral response: OAS and IFIT5 expression levels were commonly upregulated after infection in the HPAI-resistant species, but not in the HPAI-susceptible species or species with unknown sensitivity to HPAI. In addition, upregulation ratios of OAS expression at 6 h post-infection and of OAS and IFIT5 at 12 h post-infection were significantly higher in the resistant species than in the susceptible species. We conclude that IFIT5 and OAS could be genetic markers for HPAIV susceptibility, and that Okinawa rail and Japanese white stork are likely susceptible to HPAIV, indicating the need for their conservation and protection against HPAIV infection.

Keywords Avian flu, RNA-seq, Fibroblast, Wild birds, Endangered species

The influenza virus, characterized by a negative-sense, single-stranded RNA genome divided into eight segments, has historically caused frequent pandemics with a considerable impact on humanity[1]. In addition to infecting humans, the virus affects the poultry industry. Highly pathogenic avian influenza (HPAI) poses a significant global threat[2]. Recognized reservoirs of avian influenza (AI) include waterfowl, gulls, and shorebirds[3,4]. AI viruses are extensively found in wild bird populations, many of which have been identified as low-pathogenicity AI viruses (LPAIVs). Although LPAIVs are commonly isolated from free-living aquatic birds, they typically have minimal to no detrimental effects on their hosts[2]. However, there is a risk that LPAIVs can mutate into highly pathogenic avian influenza viruses (HPAIVs), leading to lethal infections in various bird species. HPAIV are thought to have evolved in poultry farms, with wild waterfowl acting as the ancestral hosts of LPAI[5]. Notably, the transmission of HPAIV from wild birds, including migratory species, to poultry has been confirmed[6]. Consequently, it is crucial to investigate the distribution of HPAIV strains and sensitivity of wild birds to this virus.

Over the past several years, HPAI caused by H5 N1 viruses, particularly those belonging to Clade 2.3.4.4b, has resulted in a global panzootic on an unprecedented scale, affecting millions of wild and domestic birds worldwide[2,7]. HPAIV frequently causes mass poultry mortality. Moreover, this virus has been implicated in widespread fatalities in wild bird populations. Documented instances include mass death of bar-headed geese (*Anser indicus*) in China[8], several species of waterfowl, such as the Eurasian wigeon (*Mareca penelope*), tufted duck (*Aythya fuligula*), black-headed gull (*Chroicocephalus ridibundus*), and Eurasian magpie (*Pica pica*), in Europe during 2016–2017[9], and Great white pelicans (*Pelecanus onocrotalus*) in Senegal in 2021[10]. Additionally, raptors and storks that are susceptible to HPAIV have been reported to succumb to infections in the US and Thailand[11–13]. Remarkably, mass mortality events among crane species have been observed in 2022[14], underscoring the susceptibility of some previously considered "HPAI-resistant" species to emerging strains

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within the Clade 2.3.4.4b. These instances highlight the broad host range of H5 N1 HPAIV and raise concerns about potential species-specific susceptibility or resistance mechanisms. Such mechanisms likely involve the innate immune response, which, if better understood, could help predict or mitigate the risk of outbreaks.

Although the impact of HPAI on wild birds is a global concern, its effects can be particularly devastating in specific regions such as Japan, where targeted surveillance and control efforts are ongoing. In Japan, HPAI outbreaks in poultry have been confirmed periodically, and the first mass poultry deaths owing to HPAIV were reported in 2004. In 2020, the year with the worst HPAI-caused damage to poultry in Japan, more than 7 million poultry birds were culled. HPAIV has been detected in at least 35 species of wild birds in Japan from 2008 to 2023[15]. Therefore, Japanese wild birds are also at risk of mass death, and methods to assess their sensitivity to HPAIV are needed to protect them from HPAIV infection.

The innate immune system is the first line of defense against viral infections. Previous studies have identified several antiviral signaling pathways in the innate immune system of chickens. The major viral defense mechanisms are retinoic acid-inducible gene I (absent in chickens and some bird species) and Toll-like receptors[16], which recognize RNA viruses, and interferon-stimulated genes, interferons, and proinflammatory cytokines, which protect against viral infections. HPAIV infection of avian species significantly alters expression levels of these antiviral genes[17]. In addition, Huang et al. report that in ducks (*Anas platyrhynchos*), a primary natural host of AI, defensins and BTNL genes may be involved in the host immune response to AI in ducks[18]. Thus, while attempts to eliminate HPAIV infection are based on various innate immune responses, it remains unclear what differences in expression of these antiviral genes exist between ducks, chickens and other avian species, which may play a role in susceptibility to HPAI. Additionally, mallards coexist with AIs and are thought to be tolerant to HPAI rather than resistant, and their tolerance mechanisms may differ from those of other HPAI-resistant birds. Studying experimental HPAIV infections in live wild birds presents several challenges. Experimental HPAIV infections require the use of live rare birds, making multiple experiments difficult or almost impossible. To solve these problems, we established a method to evaluate the susceptibility of cultured cells from rare avian species to HPAIV infection. In this study, we explore marker genes for the assessment of HPAIV susceptibility in wild birds by conducting infection experiments on avian cells conserved in cryopreservation project in Japan and analyzing their gene expression.

Results

Sequencing and analysis results

All avian cells were successfully infected with the HPAIV. TCID50 was not determined for the pigeon cells as all cells were confirmed dead 48 h post-infection. The TCID50 values of the cell supernatants ranged from 2.2 to 3.7 in avian species other than the pigeon (Table 1). RNA-seq was successful for all samples. The number of paired-end reads per sample ranged from 16.9 to 308.6 million, which was sufficient for the gene expression analysis. The number of reads per bird species is listed in Table 1. Following de novo assembly using Trinity and estimation using Transdecoder, the number of CDS regions for each sample set ranged from 114,124 to 399,028 (Table 2). The number of upregulated and downregulated DEGs varied among bird species, ranging from 90 to 1,207 and from 70 to 1,200, respectively (Table 2).

To examine whether common Pathways between bird species are variable in HPAIV infection, KEGG (Kyoto Encyclopedia of Genes and Genomes)pathway analyses of DEGs (Differentially Expressed Genes) following infection with HPAIV were performed in cells from all bird species tested, but no common expression profiles were identified between cells from birds with similar sensitivity to the infection.

Comparing DEGs among bird species

A search for commonly upregulated or downregulated DEGs in resistant birds showed that the IFIT5, OAS, and HERC3 genes were commonly upregulated, whereas no genes were commonly downregulated (Figs. 1 and 2). The upregulation ratios of IFIT5, OAS, and HERC3 were calculated from TPM (Transcripts Per Million) values.

	logTCID50/ml (Inf48)	Number of reads processed			
		CT6	Inf6	CT12	Inf12
Chicken (*Gallus gallus*)	3.7	202,845,568	50,112,307	173,877,636	37,325,215
Mountain hawk-eagle (*Nisaetus nipalensis*)	3.1	16,936,603	19,132,555	22,531,183	21,673,146
Northern goshawk (*Accipiter gentilis*)	2.3	42,070,972	34,572,447	45,185,775	51,054,603
Peregrine falcon (*Falco peregrinus*)	2.5	38,910,990	31,526,422	40,219,377	35,266,828
Golden eagle (*Aquila chrysaetos*)	2.2	30,262,790	44,526,804	44,995,719	39,798,985
Rock pigeon (*Columba livia*)	NT*	308,569,660	47,326,844	128,172,059	37,899,696
Hooded crane (*Grus monacha*)	2.4	36,417,842	81,569,838	30,407,396	40,118,803
White-necked crane (*Grus vipio*)	3.6	35,845,420	35,407,516	35,454,695	33,522,332
Japanese crane (*Grus japonensis*)	2.6	93,823,022	38,450,897	95,072,195	33,976,762
Okinawa rail (*Rallus okinawae*)	2.2	43,452,816	43,752,918	34,722,858	199,884,890
Japanese white stork (*Ciconia boyciana*)	2.5	39,669,650	45,470,285	337,329,497	92,827,202

Table 1. Number of paired-end reads processed in RNA-seq analysis in each dataset. Inf48, post-infection 48 h; CT6, control 6 h; CT12, control 12 h; Inf6, post-infection 6 h; Inf12, post-infection 12 h. *: Not tested.

	CT6 vs. Inf6			CT12 vs. Inf12		
	CDS	Upregulated	Downregulated	CDS	Upregulated	Downregulated
Chicken (*Gallus gallus*)	273,245	140	87	280,852	254	130
Mountain hawk-eagle (*Nisaetus nipalensis*)	114,124	115	150	133,364	273	315
Northern goshawk (*Accipiter gentilis*)	154,237	174	237	164,448	133	141
Peregrine falcon (*Falco peregrinus*)	147,163	1207	1200	180,213	160	242
Golden eagle (*Aquila chrysaetos*)	170,457	228	251	178,657	132	164
Rock pigeon (*Columba livia*)	309,331	191	108	235,032	138	71
Hooded crane (*Grus monacha*)	197,726	90	159	156,357	209	304
White-necked crane (*Grus vipio*)	145,286	321	817	157,837	104	142
Japanese crane (*Grus japonensis*)	231,248	364	237	227,145	197	129
Okinawa rail (*Rallus okinawae*)	204,373	198	234	320,437	92	70
Japanese white stork (*Ciconia boyciana*)	191,268	435	252	399,028	130	67

Table 2. Number of CDSs and DEGs. CDS, coding sequence; CT6, control 6 h, CT12, control 12 h; DEG, differentially expressed gene; Inf6, post-infection 6 h; Inf12, post-infection 12 h.

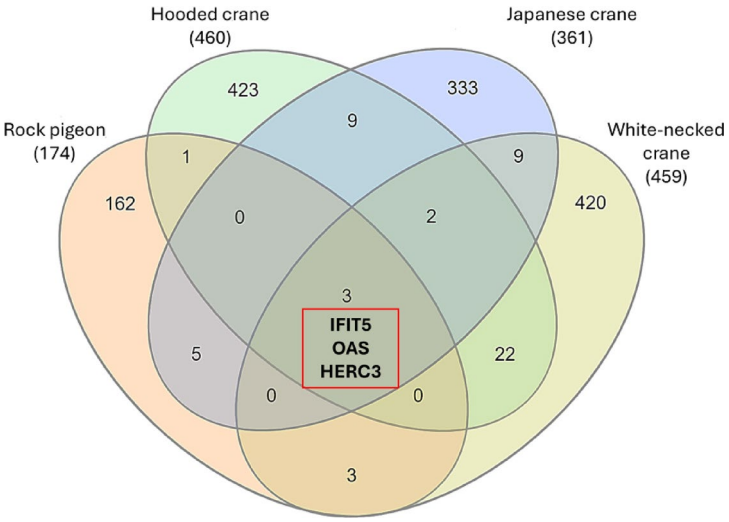

Fig. 1. Venn diagram of the genes whose expression was significantly upregulated in cells from the four HPAI-resistant birds. The three genes enclosed in the central red square were commonly upregulated in the four resistant bird species tested. HERC3, HECT and RLD domain containing E3 ubiquitin protein ligase 3; IFIT5, interferon-induced protein with tetratricopeptide repeats 5; OAS, oligoadenylate synthase.

Comparison between resistant and susceptible birds revealed that IFIT5 was significantly upregulated at 12 h post-infection, whereas OAS was significantly upregulated at both 6 and 12 h post-infection in resistant birds (Table 3; Fig. 3). Despite HERC3 was also upregulated in the resistant birds, its upregulation ratio was low and was not significantly different from that in the susceptible birds. In addition, the HERC3 transcript was not detected in the control cells at 6 h (CT6) peregrine falcon sample. Samples from Okinawa rail and Japanese white stork, i.e., species with unknown susceptibility to HPAI, showed little or no upregulation of the OAS, IFIT5, or HERC3 genes after infection.

Discussion

In the number of DEGs at 6 and 12 h after infection (Inf6 and Inf12), more genes were upregulated in the CT6 vs. Inf6 comparison in peregrine falcon, and more genes were downregulated in the CT6 vs. Inf6 comparison in white-necked crane and peregrine falcon. It is unclear whether increases or decreases in the number of DEGs are directly related to viral infection. The relationship between the number of DEGs and stress on cells will need to be investigated in the future.

IFIT5 and OAS were upregulated in bird species resistant to HPAIV infection. IFIT5 is known to be induced by interferons after viral infection and play a role in the inhibition of viral amplification. Previous studies have reported that transgenic chickens expressing IFIT5 were resistant to HPAIV[19]. IFIT5 was also significantly upregulated after HPAIV infection in ducks, which are resistant to HPAIV, supporting the notion that IFIT5 is involved in resistance to infections[19]. OAS is also induced by viral infections. The OAS protein synthesizes 2′,5′-oligoadenylic acid that activates RNase L. This endonuclease inhibits replication by degrading intracellular

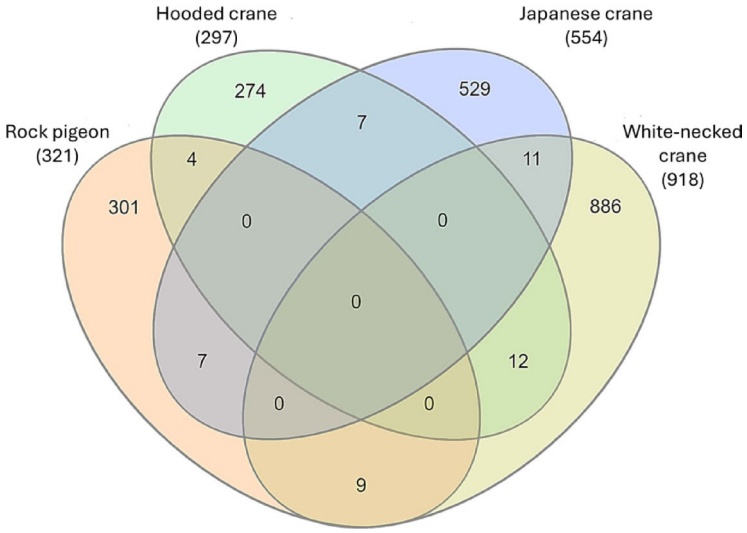

Fig. 2. Venn diagram of the genes whose expression was significantly downregulated in cell lines from the four HPAI-resistant birds.

HPAIV sensitivity	Bird species	*IFIT5*		*OAS*		*HERC3*	
		CT6 vs. Inf6	CT12 vs. Inf12	CT6 vs. Inf6	CT12 vs. Inf12	CT6 vs. Inf6	CT12 vs. Inf12
Susceptible	Chicken (*Gallus gallus*)	0.31	3.58	0.49	1.38	0.99	1.23
	Mountain hawk-eagle (*Nisaetus nipalensis*)	1.37	1.05	1.10	1.18	1.54	1.38
	Northern goshawk (*Accipiter gentilis*)	0.62	8.38	0.65	7.41	0.80	3.32
	Peregrine falcon (*Falco peregrinus*)	5.36	2.34	3.87	1.79	2.20	ND*
	Golden eagle (*Aquila chrysaetos*)	0.81	1.81	1.07	1.54	0.78	1.13
Resistant	Rock pigeon (*Columba livia*)	3.18	54.98	3.05	9.78	0.95	1.17
	Hooded crane (*Grus monacha*)	2.18	16.26	5.82	27.47	5.90	3.29
	White-necked crane (*Grus vipio*)	15.38	21.58	20.19	64.49	1.62	4.35
	Japanese crane (*Grus japonensis*)	11.10	45.24	5.65	57.56	2.68	2.20
Unknown	Okinawa rail (*Rallus okinawae*)	1.05	1.17	0.69	0.89	1.10	0.98
	Japanese white stork (*Ciconia boyciana*)	1.35	1.61	1.01	1.23	0.97	1.05

Table 3. Ratios of expression levels of the IFIT5, OAS, and HERC3 genes at different experimental points. *: HERC3 could not be detected in the CT12 sample of peregrine falcon. CT6, control 6 h, CT12, control 12 h; HERC3, HECT and RLD domain containing E3 ubiquitin protein ligase 3; IFIT5, interferon-induced protein with tetratricopeptide repeats 5; Inf6, post-infection 6 h; Inf12, post-infection 12 h; OAS, oligoadenylate synthase.

viral RNA and induces apoptosis to prevent viral spread. Indeed, OAS expression was found to be upregulated after HPAIV infection in HPAI-resistant ducks, suggesting that it is involved in resistance to HPAI[20]. IFIT5 and OAS, whose protein products have antiviral effects, tended to be upregulated in resistant bird species at 6 and 12 h post-infection, suggesting that these two genes are likely involved in the antiviral response in the early stages of HPAIV infection. HERC3 was also upregulated in the resistant species. However, the upregulation ratios of HERC3 in resistant species were lower than those of OAS and IFIT5. Furthermore, the upregulation ratios of HERC3 were not significantly different compared to those in susceptible species. HERC3 is a member of the HERC family. Another member of this family, HERC5, has been reported to be implicated in antiviral responses in mammalian cells[21]. However, to the best of our knowledge, there has been no information about antiviral activity of HERC3. Therefore, HERC3 functions in birds should be closely examined. Interestingly, a comparison of the results of the KEGG pathway analysis across bird species did not identify any common expression profiles in resistant or susceptible birds. Although expression of the RSAD2 gene, encoding viperin, was elevated in cells from several bird species, its low expression level meant that it was not selected as a DEG owing to an FDR value greater than 0.1. Phylogenetic analysis was performed using the Maximum Likelihood method and JTT matrix-based model based on the amino acid sequences of the IFIT5 and OAS genes of each bird species in MEGA11[22] (Supplementary File 1). The phylogenetic trees for IFIT5 and OAS were similar for each, and both phylogenetic trees appeared to reflect their respective host lineages. In both phylogenetic trees, cranes and raptors formed

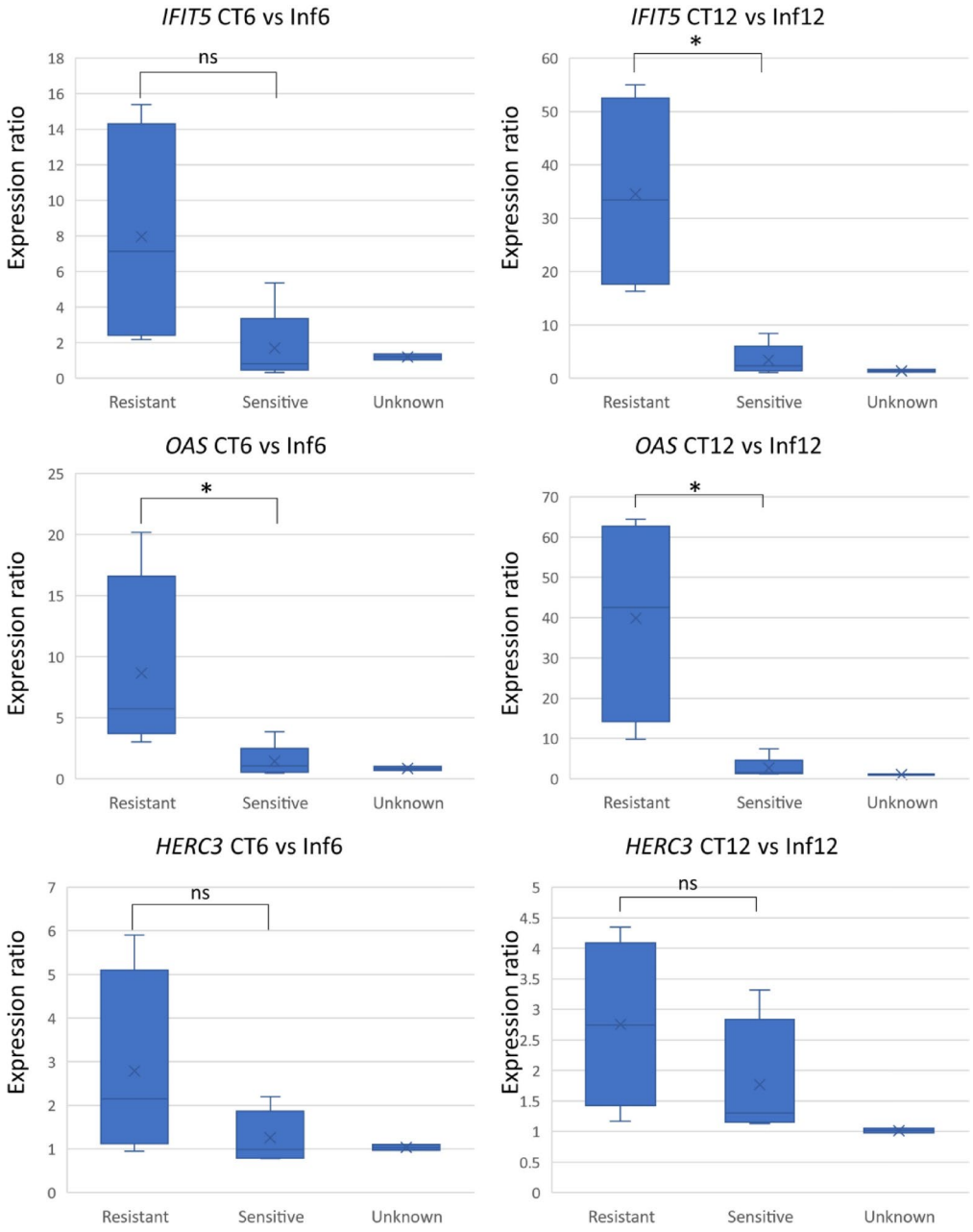

Fig. 3. Boxplots illustrate the upregulation ratios of IFIT5, OAS, and HERC3 genes in the cells from HPAI-resistant and HPAI-susceptible birds, as well as from birds with unknown susceptibility to HPAI. The asterisk (*) indicates significant differences between the two groups of HPAI-resistant and HPAI-susceptible birds at $P < 0.05$ based on the Mann–Whitney U test; "ns" denotes "not significant".

clades for each taxon and had similar amino acid sequences. Whereas no similarities in amino acid sequences of IFIT and OAS genes between resistant bird species were identified.

In Okinawa rail and Japanese white stork, IFIT5, OAS, and HERC3 expression levels were barely upregulated. Based on this gene expression profile, these species may be susceptible to HPAIV. There have been no reports of HPAIV infections in Okinawa rail or closely related birds, such as brown-cheeked rail (*Rallus indicus*) and white-breasted waterhens (*Amaurornis phoenicurus*), in Japan. This could be not because rails are resistant to HPAIV, but because they were not infected with HPAIV on a large scale owing to their distinct habitat and ecological features. In addition, previous studies have shown that the MDA5 gene is non-functional in the Okinawa rail[23]. The MDA5 gene detects RNA viruses including AIV and initiates interferon signaling, an important function in innate immunity[17]. These suggest that Okinawa rail may be highly vulnerable to AIV infection. Thus, surveillance for HPAIV should also be carried out in Okinawa rail, as this virus has been found to spread over an increasingly larger geographical area in recent years. The Okinawa rail population is estimated at around 1,500–2,000 birds and is still endangered in 2014, although its number is increasing. The risk of HPAI within

the Okinawa rail population is also expected to increase as conservation efforts aimed at the protection of this species are successful, and its population density increases. In the 2022–2023 season, a Japanese white stork died of an HPAIV infection[15]. In addition, in 2004, many closely related white storks died in Thailand in large numbers owing to HPAIV infection[12]. There are currently ~ 300 Japanese white storks in Japan, and these birds are still endangered, although conservation efforts are underway and their numbers are increasing. Storks share habitats with ducks, which are reservoirs for AI, and there is a possibility of contact between the two in reservoirs and fallow fields. In this study, the cells of the endangered Okinawa rail and Japanese white stork may be highly susceptible to HPAIV, indicating that live birds are at risk. We hope that the results of this study can be used in future conservation efforts for these endangered species.

One limitation of this study is that the use of fibroblasts alone may not provide a comprehensive assessment of the kinetics of HPAIV infection in birds. As HPAIV mainly replicates in epithelial cells, it is possible that the infection environment was not optimal. It is hoped that in the future more pathological assessment tools such as cultured epithelial cells and organoids will be established and used in non-model animals. In wild birds infected with HPAIV, various antiviral responses are activated, such as the suppression of viral amplification by immune cells after infection or induction of cell death in infected cells. As wild birds are not vaccinated, and acquired immunity is not expected to act, innate immune function is considered to be of paramount importance for defense against viral infections. Thus, it should be useful to examine cellular responses to the early HPAIV infection in wild birds, as in the present study. Cranes were defined as resistant birds in this study, but in the 2022 season, over 1,300 cranes, especially hooded and white-necked cranes, died on the Izumi Plain and 72 cranes were positive for HPAIV[15]. The H5 N1 HPAIV strain that caused this mortality outbreak was classified under Clade 2.3.4.4. b and is a different strain from A/chicken/Yamaguchi/7/2004 used in this study and classified as 2.5[24,25]. It will be necessary to determine how different clades of the virus become highly pathogenic to cranes using crane cells and new types of HPAIVs. In addition, functional analysis of the two candidate marker genes, IFIT5 and OAS, identified in this study is expected to enhance understanding of the immune response to HPAIV in birds.

Another limitation of this study is we were unable to establish cell lines from mallards, which are commonly viewed as key reservoir hosts for AIV, particularly those of LPAIV. Based on previous studies, mallards have been considered tolerant to avian influenza, and they may possess tolerance mechanisms distinct from those observed in the resistant bird species examined in this study. Future work should therefore include the isolation and characterization of mallard-derived cells to clarify their susceptibility and immune responses to HPAI infection. We used the chicken genome to annotate wild bird CDS regions in this study, we may only be able to identify highly conserved genes and may not be able to identify mechanisms of resistance to HPAIV in wild birds. In addition, the tissue sources of the cells was not strictly standardized, making direct comparison of infection conditions challenging. It is possible that embryo-derived and skin-derived cells may differ in their gene expression and viral susceptibility, which may induce biases in their gene expression profiles. Although this is practically difficult in experiments using cells derived from wild birds, it will be necessary to standardize the tissue source, such as using skin-derived cells or embryo. Furthermore, increasing biological replication is expected to further improve the reproducibility of experiments. Lastly, the MOI was conducted under a single condition. It would be beneficial to report on the MOI under multiple conditions in future studies.

We showed that OAS and IFIT5 expression levels could potentially be used for non-or low-invasive HPAIV susceptibility assessment in wild birds. The cost of RNA-seq is decreasing, and the practice of preserving cells for wildlife conservation has been adopted in several countries. We hope that our research offers a method that allows using preserved cells to assess extinction risks and implement efficient conservation measures against infectious diseases such as HPAI.

Methods

Preparing primary bird cell

Eleven bird primary cells, prepared in our laboratory, were used in this study. The cells were isolated from the embryo of chicken (*Gallus gallus*) and Japanese white stork (*Ciconia boyciana*), and the skin of rock pigeon (*Columba livia*), mountain hawk-eagle (*Nisaetus nipalensis*), northern goshawk (*Accipiter gentilis*), peregrine falcon (*Falco peregrinus*), golden eagle (*Aquila chrysaetos*), hooded crane (*Grus monacha*), white-necked crane (*Grus vipio*), Japanese crane (*Grus japonensis*), and Okinawa rail (*Rallus okinawae*). Since the cells used in this study were cryopreserved at NIES under the genetic resource banking project known as the Timecapsule Project[26], ethical review and approval were waived. The detailed information and cell images are provided in the Supplementary File 2. The primary bird cells were adjusted by the method of Hagiwara et al.[27], i.e., the tissues were cut into 1–2 mm pieces using scissors. The culture medium Kuwana's Avian Medium-1[28] was added to the tissues and mixed well in a culture dish. The mixtures were transferred to a cell culture flask. The samples were incubated at 38 °C with ambient air conditions.

All the primary cells were morphologically fibroblast-like (Supplementary File 2). Based on the previous cases of HPAIV infection and experimental studies, these bird species were classified into three groups: HPAI-resistant (rock pigeon, hooded crane, white-necked crane, and Japanese crane), HPAI-susceptible (chicken, mountain hawk-eagle, northern goshawk, peregrine falcon, and golden eagle), and species with unknown susceptibility to HPAI (Okinawa rail and Japanese white stork). In this study, species that do not show a high mortality rate when infected with HPAI were defined as 'HPAI-resistant', species that have a high mortality rate when infected with HPAI were defined as 'HPAI-susceptible', and species that have few cases of HPAI infection and cannot be determined were defined as 'unknown susceptibility to HPAI. 'HPAI-tolerant' is defined as a species that co-exists with HPAI, such as mallard ducks. Rock pigeons are considered HPAI-resistant, as they were either unable to establish infection or, if infected, had a subclinical infection in experimental HPAIV infection challenges[29]. Before the 2022 season, cranes were deemed HPAI-resistant, as the HPAIV seroprevalence was low, and no mass

mortality occurred, despite the fact that large numbers of cranes fly to the Izumi Plain, where HPAIV was isolated from the environmental water[30]. In addition, during the 2010–2011 season, only seven cases of H5 N1 HPAIV infection in cranes were identified and no mass deaths were reported despite infected individuals being in high-density herds for several months[31]. Israel has similarly experienced multiple H5 HPAIV pandemics before 2022, but there were no reports of mass mortality in cranes, and the fact that crane mass mortality occurred after 2022, when HPAIV changed mainstream to Clade 2.3.4.4b[14]. Similarly, although fatal infections in pigeons with Clade 2.3.4.4b HPAI have been confirmed since 2022[32], they were previously considered to be "dead-end" hosts resistant for AI[33]. Thus, rock pigeons, hooded cranes, white-necked cranes, and Japanese cranes were classified as HPAI-resistant in this study. Chickens and many raptor species are classified as HPAI-susceptible, as their deaths from HPAI have been reported worldwide[11,34–38]. Storks (Ciconia sp.) and rails (family Rallidae) have not been evaluated for the sensitivity to the HPAIV infection; therefore, Okinawa rails and Japanese white storks were classified as having unknown HPAI susceptibility. The HPAIV strain used in this study was A/chicken/Yamaguchi/7/2004 (H5 N1) in Clade 2.5, which is known for its high pathogenicity and association with mass chicken deaths in Japan[24].

Experimental infection and RNA extraction

The avian cell cultures were maintained in Kuwana's Avian Medium-1 at 38 °C in an incubator. The avian cells were cultured in 6-well plates and the experiment was initiated when cell proliferation reached over 80% confluence. The starting point was designated as 0 h, at which time the cell count was determined. Based on this cell count, the viral infection dose was calculated. For virus infection, cells were exposed to a viral suspension with a multiplicity of infection (MOI) of 0.1 for 1 h at 38 °C. Subsequently, the viral suspension was discarded, and the cells were rinsed with phosphate-buffered saline. Thereafter, the infected cells were maintained in Kuwana's Avian Medium-1 at 38 °C in an incubator. The experiment was conducted with three technical replicates. Control cells examined at 6 and 12 h intervals were labelled CT6-1, −2, −3, and CT12-1, −2, −3, respectively. Similarly, cells infected at 6 and 12 h post-infection were labelled as Inf6-1, −2, −3, and Inf12-1, −2, −3, respectively. In other words, 12 wells of cell culture per species were used for RNA sequencing in this study. In addition, to confirm the infection, cells infected at 48 h post-infection were prepared and the supernatant was measured TCID50 using MDCK cells as described previously[39]. The experimental infection was conducted at BSL3 facilities at Hokkaido University.

RNA was extracted from the CT6, Inf6, CT12, and Inf12 samples of each bird species using a NucleoSpin RNA extraction kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. Following extraction, RNA purity was ascertained using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and RNA concentration was measured using a Qubit fluorometer (Thermo Fisher Scientific).

Library preparation and RNA-Seq

Libraries were prepared using TruSeq stranded mRNA following the manufacturer's instructions. For each sample (i.e., each well serving as a technical replicate), we prepared and sequenced separate libraries to preserve replicate-specific variability and ensure reproducibility. No pooling of RNA from different replicates was performed at this stage. Sequencing was performed using a HiSeq X system (Illumina, San Diego, CA, USA) by Macrogen Japan Corp. (Tokyo, Japan).

De Novo assembly, Quasi-Mapping, and comparisons of gene expression levels

Raw read data were quality-checked using FastQC v0.11.9[40]. Quality-checked reads were then assembled de novo for CT6, Inf6, CT12, and Inf12, using Trinity v2.14 with trimmomatic option[41]. The coding sequences (CDSs) in the assembled files were estimated by Transcoder v. 5.5[42] using all avian protein databases downloaded from RefSeq. CDS regions were extracted in the FASTA format and annotated by BLAST2GO[43] with blastx using RefSeq chicken protein sequences. The checked reads were quasi-mapped to the CDS regions in each condition using kallisto v. 0.46.1[44], the number of mapped reads was discharged, and the transcripts per million (TPM) value was calculated from the number of mapped reads.

The read count data were combined with the annotation results. CT6 vs. Inf6 and CT12 vs. Inf12 comparisons in each bird species were done with DESeq2 using iDEP96[45], and differentially expressed genes (DEG) with a false discovery rate (FDR) of less than 0.1 were extracted. In addition, the KEGG pathway analysis using iDEP96[46,47] was also performed to search for common expression profiles between bird species. The extracted DEG lists were combined for each resistant bird species, and duplicate and non-annotated genes were deleted. DEGs that were commonly up or downregulated in resistant birds were visualized using interactive Venn diagrams[48]. DEGs that were commonly up- or downregulated in the resistant birds were also searched for in the HPAI-susceptible birds and those with unknown susceptibility, and their respective TPMs were calculated to determine the extent to which the gene was up- or downregulated by the infection. Based on the TPM, the up- or downregulation ratio of each DEG was calculated and statistically analyzed using the Mann–Whitney U test between the resistant and susceptible bird species. Differences were considered statistically significant if $P < 0.05$.

Data availability

All raw data (FASTQ files) are available in the NCBI for Biotechnology Information SRA database (PRJDB17017).

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Author contributions

Conceptualization, M.O. and Y.S.; methodology, K.N. and A.S.; formal analysis, K.N; investigation, A.S.; resources, M.O. and Y.S.; data curation, K.N. writing—original draft preparation, K.N.; writing—review and editing, M.O and Y.S.; supervision, M.O and Y.S.; funding acquisition, M.O. and Y.S. All authors have read and agreed to the published version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Ethics statement

Ethical review and approval were waived for this study because the cells used in this study were cryopreserved at NIES under the genetic resource banking project, known as the Timecapsule Project[26].

Additional information

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